



SPECIFICATION

TITLE OF THE INVENTION

An antiviral therapeutic drug with therapeutic effects on syndromes

BACKGROUND OF THE INVENTION

Field of invention

The present invention relates to therapeutic agents for viruses more particularly, it relates to therapeutic agents with a molecular weight of less than 450,000, containing extracts from certain mushrooms as active ingredients and having therapeutic effects on syndromes possibly caused by a species of retroviruses, HIV, ALT (adult T-cell leukemia) and pathogenic bacteria. For further details, the mushroom used in the present invention is a specific mushroom which belongs to the class *Basidiomycetes*, namely Kabanoanatake [scientific name: *Fuscoporia obliqua* (Fr.) Aoshima], the mycelium extracts thereof constitute the major active ingredients with a molecular weight of less than 450,000 of the therapeutic agents of the present invention. In addition, the present invention has had extreme difficulty in production of the active ingredients obtained from artificial culture of Kabanoanatake. The present invention also concerns the artificial culture to obtain the present invention in association with which steady manufacturing procedures in the artificially cultured product could be developed.

Description of related art

Recently, AZT, DDI and other agents are known as a species of retroviruses, HIV (AIDS virus) inhibitors. However, these chemical agents have problems such as side effects on the human body as well as HIV drug resistance, and mushroom extracts have been utilized as anti-HIV agents to avoid such problems. There is a published document (1), as an example, of Japanese patent documents before examination, Showa Era 63-316734. This document contains a description of 'anti-HIV agents' of which the active ingredients are polysaccharides or protein polysaccharides extracted from *Basidiomycetes*, but the experiment describes only three kinds of samples; *Ganoderma lucidum*, *Flammulina velutipes* *Auricularia fuscusuccinea* as the main examples of *Basidiomycetes*. As an example (2) of published Japanese patent documents before examination, there is also a Heisei Era 2-134325, in which an HIV therapeutic agent comprising one constituent extracted from mycelium cultures of *Basidiomycetes* is

published under the title, "HIV therapeutic agent and production method thereof". In the public example (2), as the range to obtain *Basidiomycetes*, *Lentinus edodes*, *Coriolus Versicolor*, *Pleurotus ostreatus*, *Flammulina velutipes*, *Ganoderma lucidum*, *Grifola frondosa* are listed, but the embodiment described in it is limited to *Lentinus edodes*.

Looking through these published examples, in published example (1), 10g of freeze-dried extracts of *Ganoderma lucidum*, *Flammulina velutipes*, *Auricularia fuscusuccinea* were respectively dissolved in 100 mg of distilled water to be used at a concentration of 1mg/ml, and in published example (2), brown powder, freeze-dried extract from *Lentinus edodes* was investigated at concentrations of 0.1, 0.25, 0.5mg/ml, but actual suppressive effect on syncytium formation was observed only in the relatively high concentration of 0.5mg/ml. This indicates that, in short, extracts from the mycelium of mushrooms of the commonly known varieties such as *Lentinus edodes*, *Ganoderma lucidum*, *Flammulina velutipes*, *Auricularia fuscusuccinea* have anti-HIV effects, but don't have a sufficient degree of activity. In proof of that, in Case 1 of clinical tests of the published example (2) ((9) clinical test for HIV patients of the published example (1)), there is a description of a patient in whom T4 cell count increased from 1250/mm³ to 2542/mm³, but patients with an advanced HIV disease usually have a lower than normal T4 cell count (i.e. 300/mm³). On the contrary, the fact that a patient who has a T4 cell count of 1250/mm³ recovered only leads to a conclusion that anti-HIV activity of the active ingredients (*Lentinus edodes*) is low. In fact, Case 2 of the same published example describes a patient with T4 cell count of 822 that died from complications of pneumonia, in spite of being orally administered the active ingredients of *Lentinus edodes*.

Furthermore, there is no prior research suggesting that active substances derived from mushrooms are effective not only against HIV but also against pathogenic bacteria.

Usually, antibiotics have been used as a remedy against pathogenic bacteria, which had some effects on the bacteria, but due to cumulative use, drug-resistant strains are increasing which causes new problems. For instance, though *E. coli* bacteria, an indigenous strain, is generally said not to have pathogenicity, among them there appeared *E.coli* bacteria that can cause severe disease such as *Escherichia coli* O157, which has recently become a topic of widespread concern and can be treated significantly by conventional therapy. In these circumstances, the advent is anticipated of new anti-bacterial agents and health food ingredients that replace or supplement the

conventional antibiotics or incorporate with them.

The inventor has found so far that the extracts from Kabanoanatake [scientific name: *Fuscopia oblique* (Fr.) Aoshima] have anti-tumor and anti-HIV effects and filed patent documents to the Japanese Patent Office (Tokugan Hei 1-114665 'anti-tumor agents and anticancer food that may be administered orally and production method for mycelium', which was afterwards awarded a Japanese patent, No. 114665) and Tokugan Hei 8-23208, 'anti-HIV agents' ('Tokugan' mean a patent application. 'Hei' is the abbreviation of (Heisei) which is the current Emperor's era that started in 1989). From having sought further benefits on the basis of these observations, it was confirmed that the Kabanoanatake extracts contain great quantities of LPS (Lipopolysaccharide) and pseudo-humic acid (humic like polyphenol complex) and triterpenes which has a high content of methoxyl groups including also monolignin with a molecular weight of less than 450,000. From there, it was found that the Kabanoanatake extracts are therapeutic effective in cancers, microbe-related syndromes such as HIV, ATL (adult T cell leukemia virus) and pathogenic bacteria, especially *Escherichia coli* O157. To compare the effectiveness of the present invention with extracts from another mushroom (*Lentinus edodes*), clinical reports of actual HIV patients receiving doses of *Lentinus edodes* extract were examined. According to these reports, the daily required dose of *Lentinus edodes* extract for HIV patients was 9g. Additionally, the inventor's former anti-HIV Kabanoanatake extract, previously patented in Japan (The present invention uses a Kabanoanatake extract, referred to herein as the primary processed matter. Discussed below.), was dosed at 3g per day. However, the required daily dose of the present invention is 0.5-1.5g per day. Even if the present invention is an extract from the same Kabanoanatake, its molecular weight is below 450,000, and it exhibits remarkable and exceptional and antiviral capabilities and preventive or therapeutic effects on syndromes.

Next, through the use of a test for HIV-blocking, various comparisons of the present invention with other substances will be made to demonstrate its superiority. Then, the varied effects of the present invention will be discussed, including their application to relieving a variety of syndromes. These unusually beneficial properties, as well as methods for practical applications thereof, will be fully disclosed together with the results of experiments based on the fundamental scientific principle. As examples, the present invention suppresses carcinogenic promotion and thus exhibits anti-cancer properties, acts in an SOD-like manner and thus has the potential to treat conditions such as atopic dermatitis, inhibits the agglutination of fat and thus has the

potential to be used in treatments for obesity or diabetes, and increases physical strength, has anti-influenza properties, and anti-VRE (ancomycin resistant enterococci) effects. The anti-syndrome effects of the present invention are specified while the relationship between the physical effects and actual medical treatments are delineated. Then, the use of the present invention as an antiviral therapeutic drug with anti-syndrome effects is described, as well as the effects on uniformity of symptoms. A collapse into immunodeficiency is a common occurrence in humans at the time of death. It is crucial to retain the highest possible functioning of the immune system and to revitalize the nervous and physical systems. To achieve this, it is necessary to deal with each respective issue (disease). However, when each individual issue (disease) is fought vigorously, the result of death due to reactions to or side effects of medication frequently arises. The fundamental idea behind the development of the present invention is the safety of the life form (human, animal, plant), with the goal of producing a single medication with a variety of effects. Therefore, the present invention exhibits unique, unexpected and unprecedented effects and strongly reinforces the essential systems of the life form. For example, when a human or animal receives a dose of the present invention, an increase in physical strength is observed for some time. Plants exhibit excellent growth. Therefore, in both healthy and sick individuals, a daily dose of the present invention helps to combat fatigue. In addition, the present invention stimulates the cerebral cortex and has positive effects on the heart. These are the basic properties of the present invention, ideas behind its development, and goals for its use. Next, a detailed review is presented, following the outline given above. The present invention clearly surpasses my prior art techniques and represents one small step towards a better future.

SUMMARY OF THE INVENTION

The present invention provides therapeutic agents effective in preventing various syndromes caused by microbes by killing HIV and bacteria such as enteropathogenic *Escherichia coli*, particularly *Escherichia coli* O157 and MRSA (methicillin resistant *Staphylococcus aureus*, or at least inhibiting the growth of them, wherein the active ingredients thereof are the extracts from Kabanoanatake [*Fuscoporia obliqua* (Fr.) Aoshima].

Kabanoanatake belongs to *Basidiomycotina*, *Hymenomycetidae*, *Aphylllophorales*, *Hymenochaetaceae*, *Kabanoanatake* [scientific name: *Fuscoporia obliqua* (Fr.) Aoshima], and it is classified as growing naturally on the trunks of birch trees such as

white birch (*Betula platyphylla* Sukatchev. var. *japonica*) and *Betula ermanii*, which forms black sclerotia like a coal-like mass. Its sclerotia may grow up to about 20 cm long, originally known as evil fungi (form of cancer) in regard to birch trees such as white birch. However, the inventor (Kazuo Sakuma) embarked on research by noticing the strong life force possessed by Kabanoanatake (common name: Chaga), whereby it has been found that the hyphae extracts thereof may have anti-cancer effects and possess inhibitory effects against hepatitis B virus, influenza virus, HIV, enteropathogenic *Escherichia coli*, especially *Escherichia coli* O157, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, MRSA (methicillin resistant *Staphylococcus aureus*), and clostridia of gas gangrene.

Through the present invention, it was shown that natural and cultured Kabanoanatake are both equally effective. However, it is preferable to make use of Kabanoanatake grown in artificial cultures when required in large amounts, since natural Kabanoanatake cannot be taken easily because it can be obtained in only a small amount from one stock and its yield is low. There are several methods for artificial cultures, but sawdust culture and liquid culture are preferable. It is easy to describe that it can be cultured, but it has been very difficult to exhibit useful effects under any culture condition. The present invention resolved this problem by a new device. Furthermore, hyphae and its secretions were proliferated artificially by planting Kabanoanatake hyphae on the green wood of birch trees, or formed sclerotia by growing this hyphae so it can be utilized effectively in the present invention. Artificial Kabanoanatake inoculated hyphae are in line with the natural form. The effective development of this artificial culture was achieved by the inventor for the first time in the world after long research.

The liquid culture method of the present invention is described here. Production of the active ingredients of Kabanoanatake by artificial culture has been very difficult. It was frequently impossible to collect active ingredients after selecting the appropriate hyphae, even under appropriate culture conditions. The active ingredients have not been produced only by extending the hyphae of Kabanoanatake. In addition, the production was difficult in winter when the environmental temperature changed. When the culture temperature decreased to 10°C from 33 or 25°C even while heating and when the culture medium encountered severe conditions for a certain period because the shaking stopped due to electric power failure, the hyphae of Kabanoanatake in liquid culture media did not produce any active ingredient. As shown in Fig. 13, because, in the control group not treated with the technique of the present application, the hyphae of

A1W-27 and A1W-4 stopped growth and became corrupt before reaching 30 days of culture period, this group was excluded. In Fig. 13, moreover, for example, A1W-27 is the hyphae culture group which was ineffective after 34 and 37 days of culture even after giving the technique of the present invention (lignin substances), which exhibited effective effects (production of active ingredients) on AIDS viruses on Day 70 of culture because of addition of lignin substances to the culture medium in advance. As described above, artificial culture of Kabanoanatake is very difficult. Since the techniques were studied and confirmed one by one along with the hyphae, a new useful technique possible to produce active ingredients was achieved.

As described in detail, a steady technique of artificial culture which has not been in previous literatures was developed by various unique devices and efforts described later. In this application, in other words, the efficacy of the primary processed matter in artificial culture to obtain the present invention was realized (by introduction of new techniques), and the present invention was established based on the techniques. This liquid culture consisting of inoculating the seed fungi from precultured Kabanoanatake in a liquid medium (culture solution) containing a mixture of a carbon source selected from the group of malt, glucose, saccharose and starch as well as peptone and yeast extract with water and phosphate buffer, incubating at a temperature of around 20 to 30°C, preferably at 25°C, for a short period of 20 to 49 days, or in some cases for a long period over 100 days, and growing the hyphae by shake culture method. For the liquid medium of the present invention, suitably adding wood constituents, to be more specific, one or more substances selected from lignin sulfonic acid, lignosulfonic acid sodium salt, lignosulfonic acid sodium salt acetate, lignin alkali, lignin organosolv, lignin organosolv acetate, 2-hydroxypropyl ether, lignin hydrolytic, hydroxymethyl derivative, lignin organosolv propionate, betulin (betulinic acid), or lignin salts, the growth state, yield and potency of hyphae can be improved. The growth state of hyphae is determined based on the density of the black pigments developed in hyphae and in the culture medium, nutrients in the culture medium, particularly decrement of carbon source, protein quantity secreted by hyphae in the culture medium, and pH of the culture medium.

The harvest (collection of the active ingredients) is done by determining the time of formation of the active ingredients having sufficient anti-HIV activity, which was produced by hyphae. One of the practical harvest methods for the active ingredients by liquid culture method concerning the present invention, is to extract mycelium along with culture solution (including secretions of the hyphae) in hot water, filtrate them, filtrate the filtrates again where necessary, and to subject to molecular sieve to

fractionate the fraction with a molecular weight of 450,000. Consequently, the fraction containing active ingredients is obtained. Or, it is to dry the fraction described above by vacuum-freeze to get the active ingredients in a powder form. The state (condition before fractionation) of the components extracted from the material of Kabanoanatake is referred to as the primary processed matter in the whole text of the specification of this application as a matter of convenience, and the applicant will explain the nature of Kabanoanatake based on the primary processed matter while explaining the summary of the past research in comparison with the invention of this application. In addition, the applicant will explain the method to obtain the active ingredients with a molecular weight of 450,000, which is much better than the primary processed matter itself. The invention surpasses prior techniques and production of substances. Hereinafter, the applicant will clarify the innermost power of Kabanoanatake and explain its summary. As a matter of convenience, the active ingredients with a molecular weight of less than about 450,000, which are effective as antiviral substances, and were obtained by subjecting the primary processed matter to molecular sieve, are referred to as the present invention.

In the culture process, the large part (about 90%) of the active ingredients having anti-HIV activity, etc. is contained in the culture solution, and the active ingredients extracted from cultured mycelium are relatively small amounts (about 10%). Liquid medium (culture solution) is not only a source of nutrition to grow hyphae, but also a place to store the active ingredients secreted from the growing hyphae.

Whether the active ingredients a primary processed matter obtained from Kabanoanatake and the present invention with the molecular weight of less than 450,000 have anti-HIV activity or not was determined by the following test procedure, which does not constitute the present invention by itself.

(1) Test procedure

The test to measure cell damage was conducted by using MT-4 cells. 96-well microplates were arranged in eight rows, A-H (sample numbers) and twelve columns, 1-12 (well numbers). 100 μ l of dilution (cell culture solution) was placed in each of the wells, and an additional 100 μ l of sample A was added to well A1 and 100 μ l of the solution was thrown out. Then, two-fold serial dilution was carried out in each of the subsequent wells from A2 to A12. Namely, starting from 1: 2 dilution in A2 well, the dilution ratio of the sample was serially increased as follows; 1: 4 in A3, 1:8 in A4 and finally, 1:2048 in A12. The same serial dilution process was repeated for the samples, B

to H, from well B1 to H12. After all the required samples were diluted, 100 μ l of dilution in each well was thrown out. 100 μ l of suspension of cultured cells (MT-4 cells) incorporated with HIV was placed into all the wells so that the total liquid amount in each well became 200 μ l. The cells had been cultured, and after that the inhibition level for HIV was observed.

(2) Numeration

1) The effective dose for inhibiting HIV is calculated as follows: in the case of inhibition against the HIV virus observed in the well number 8 (at final dilution, 1:128), where the specimen stock solution is counted to be $100\mu\text{l} \div 128 = 0.78\mu\text{l}$, which is converted into $0.78 \times 10 = 7.8\mu\text{l}$ per 1m dilution; The effective dose for inhibition is expressed as 7.8 $\mu\text{l/ml}$.

2) Amount of cell damage

The samples were diluted in the same manner as the ones in the above inhibition test for HIV to measure the amount of cell damage. Cell suspension culture (MT-4), to which the HIV virus was not added, was placed in each well, and the liquid amount where cells died (dilution ratio) was measured and represented numerically. This indicates the safety of the said samples in biological cells.

BRIEF DESCRIPTION OF DRAWINGS

An embodiment of the primary processed matter and the present invention will be described below with reference to the accompanying drawings.

Fig.1 is a graph showing the extracts from the black part of Kabanoanatake which are effective as anti-HIV agents (black circle) in the primary processed matter, Kabanoanatake liquid culture extracts (white circle) in the primary processed matter and the inhibition ratio for syncytium formation (ordinate) by conventional AZT (black triangle) in varied consistency (abscissa), by comparison.

When 0.1 $\mu\text{l/ml}$ of the extracted solid content from both the black part of Kabanoanatake and the cultures of Kabanoanatake is used respectively, each solution concentration is 350 ng (nanogram).

Fig. 1b is a graph showing the inhibition rate (ordinate) against the formation of giant cells of AIDS virus of the extracts from the black part of Kabanoanatake which are effective as anti-HIV agents (black circle) in the present invention, Kabanoanatake liquid culture extracts (white circle) and conventional AZT (black triangle) in varied

consistency (abscissa), by comparison. When 0.1 $\mu\text{l/ml}$ of the extracted solid content from both the black part of Kabanoanatake and the cultures of Kabanoanatake used respectively, each solution concentration is 350 ng (nanogram).

Fig.2 is a graph showing the inhibition ratio for HIV infection (ordinate) in varied consistency (abscissa) regarding the extracts of the black part of natural Kabanoanatake (black circle), extracts by sawdust cultures (white circle), hyphae grown in liquid culture and dried by heating (black quadrangle), viable hyphae grown in liquid culture and viable hyphae dried by heating (white triangle), filtrates grown in liquid culture and dried by heating (black triangle), which are anti-HIV agents concerning the primary processed matter. Each extracted solid material is 350 ng when the concentration level of the solution is 0.1 $\mu\text{l/ml}$, and it is 3500 ng when the level is 1 $\mu\text{l/ml}$.

Fig.2b is a graph showing the inhibition ratio for HIV infection (ordinate) in varied consistency (abscissa) regarding the extracts of the black part of natural Kabanoanatake (black circle), extracts by sawdust cultures (white circle), hyphae grown in liquid culture and dried by heating (black quadrangle), viable hyphae grown in liquid culture and viable hyphae dried by heating (white triangle), filtrates grown in liquid culture and dried by heating (black triangle), which are anti-HIV agents concerning the present invention. Each extracted solid material is 350 ng when the concentration level of the solution is 0.1 $\mu\text{l/ml}$, and it is 3500 ng when the level is 1 $\mu\text{l/ml}$.

Fig.3 is a graph illustrating how the primary processed matter affects the cell viability within both the non-infectious systems and the infectious systems in varied consistency, with the viability (%) on the ordinate and the elapsed number of days on the abscissa.

Fig.3b is a graph illustrating how the present invention affects the cell viability within both the non-infectious systems and the infectious systems in varied consistency, with the viability (%) on the ordinate and the elapsed number of days on the abscissa. (Concentrations higher than 35 $\mu\text{g/ml}$ cannot be illustrated because it is effective.)

Fig.4 is a graph illustrating the HIV P24 antigen yield (ordinate) of the primary processed matter in the same consistency as Fig.3 and the elapsed number of days (abscissa).

Fig.4b is a graph illustrating the HIV P24 antigen yield (ordinate) of the present invention in the same consistency as Fig.3 and the elapsed number of days (abscissa). (Concentrations higher than 350 $\mu\text{l/ml}$ cannot be illustrated because it is effective.)

Fig.5 -A is a graph illustrating the pretreatment effects on HIV of Kabanoanatake, and;

Fig.5 -B is a graph illustrating the pretreatment effects on target cells of Kabanoanatake.

Fig.6 -A is a graph illustrating the correlation between the pretreatment time of target cells by the primary processed matter of Kabanoanatake and the infection inhibition efficacy, and;

Fig.6 -B is a graph showing the inhibition ratio when the primary processed matter of Kabanoanatake was added in various incubation time periods, after 1 hour pretreatment of the cells with Kabanoanatake.

Fig.6 -A2 is a graph illustrating the correlation between the pretreatment time of target cells by the present invention and the infection inhibition efficacy, and;

Fig.6 -B2 is a graph showing the inhibition ratio when the present invention was added in various incubation time periods, after 1 hour pretreatment of the cells with Kabanoanatake.

Fig.7 is a graph illustrating the suppressive effect of anti-HIV agents concerning the primary processed matter on the syncytium formation of non-infected cells that were cultured together with infected cells.

Fig.7b is a graph illustrating the suppressive effect of anti-HIV agents concerning the present invention on the syncytium formation of non-infected cells that were cultured together with infected cells.

Fig.8 is a graph illustrating the infection inhibition efficacy in varied consistency concerning the five kinds of anti-HIV agents in the primary processed matter of Kabanoanatake.

Fig.8b is a graph illustrating the infection inhibition efficacy in varied consistency concerning the five kinds of anti-HIV agents in the present invention of Kabanoanatake.

Fig.9 is also a graph illustrating the suppressive effect on syncytium formation in varied consistency concerning the five kinds of anti-HIV agents in the primary processed matter .

Fig.9b is also a graph illustrating the suppressive effect on syncytium formation in varied consistency concerning the five kinds of anti-HIV agents in the present invention.

Fig.10 and Fig. 10b is a list showing the results of the test for HIV. Fig. 10 shows the results of administration of the primary processed matter, and Fig. 10b shows the results of administration of the present invention in the same patient shown in Fig. 10.

Fig.11 is a graph illustrating perfect inhibition effects on HIV, using a strain of Kabanoanatake (AIW ro-4) in liquid culture for 62 days, under extreme conditions of diurnal incubation temperature of 33°C and night incubation temperature falling to 8°C to 10°C. Based on this epoch-making success, it was successful to collect the primary processed matter and to obtain the present invention.

Fig.12, associated with Fig.11, is a graph illustrating cell damage (which does not mean 'cytotoxicity'), concerning a strain of Kabanoanatake (AIW ro-4) in liquid culture for 62 days, under extreme conditions of diurnal incubation temperature of 33°C and night incubation temperature falling to 8°C to 10°C.

Fig.13 is a graph illustrating perfect inhibition effects on HIV in a culture medium including lignin as wood constituents in a long-term culture test, using strains of Kabanoanatake of AIW-27 and AIW-4, under extreme conditions of diurnal incubation temperature of 33°C and night incubation temperature falling to 8°C to 10°C, in addition to restricting the infiltration of oxygen. Because of the success of very difficult culture techniques, it was successful to collect the primary processed matter and to obtain the present invention.

Fig.14, associated with Fig.13, is a graph illustrating the change in cell damage in culture medium including lignin as wood constituents in a long-term culture test, by means of strains of Kabanoanatake (AIW-27, AIW-4), under extreme conditions of diurnal incubation temperature of 33°C and night severe incubation temperature falling to 8°C to 10°C, in addition to restricting the infiltration of oxygen.

Fig.15, associated with Fig.13, is a graph illustrating the change in anti-HIV effects by addition of lignin substance as wood constituents to the culture medium, in liquid culture for 34 days, using a strain of Kabanoanatake (A-2W-3), under extreme conditions of diurnal incubation temperature of 33°C and night incubation temperature falling to 8°C to 10°C. Because of the success of very difficult culture techniques, it was successful to collect the primary processed matter and to obtain the present invention.

Fig.16, associated with Fig.15, is a graph illustrating cell damage (which does not indicate 'cytotoxicity', because constituents of Kabanoanatake are derived from natural products and taken orally) by addition of lignin substance as wood constituents to the culture medium, in liquid culture for 34 days, using a strain of Kabanoanatake (A-2W-3), under extreme conditions of diurnal incubation temperature of 33°C and night incubation temperature falling to 8°C to 10°C. Because of the success of very difficult culture techniques, it was successful to collect the primary processed matter and to obtain the present invention.

Fig.17, associated with Fig.13, is a graph illustrating the change in black color tone observed at 500 nm in culture medium including lignin as wood constituents in a long-term culture test, using strains of Kabanoanatake of AIW-27 and AIW-4, under extreme conditions of diurnal incubation temperature of 33°C and night incubation temperature falling to 8°C to 10°C, in addition to restricting the infiltration of oxygen. (This is considered as the standard for the harvest of Kabanoanatake.)

Fig.18 is a graph illustrating the change in humic acid in culture medium including lignin as wood constituents in a long-term culture test, using strains of Kabanoanatake of AIW-27 and AIW-4, under extreme conditions of diurnal incubation temperature of 33°C and night incubation temperature falling to 8°C to 10°C, in addition to restricting the infiltration of oxygen.

Fig.19, associated with Fig.13, is a graph illustrating the change in lignin tannin in culture medium including lignin as wood constituents in a long-term culture test, using strains of Kabanoanatake of AIW-27 and AIW-4, under extreme conditions of diurnal incubation temperature of 33°C and night incubation temperature falling to 8°C to 10°C, in addition to restricting the infiltration of oxygen.

Fig.20 is a graph illustrating the change in protein content in culture medium in a long- term culture test, using strains of Kabanoanatake of AIW-27 and AIW-4 under extreme conditions of diurnal incubation temperature of 33°C and night incubation temperature falling to 8°C to 10°C, in addition to restricting the infiltration of oxygen.

Fig.21 is a graph illustrating the values of perfect (100%) HIV inhibition activity by strains of Kabanoanatake hyphae, A to E, cultured at the ideal temperature for cultures of around 25°C, on the 110th day of liquid culture (lignin substances as wood constituents were not added to the culture medium).

Fig.22 is a graph illustrating comprehensively the values of perfect (100%) HIV inhibition activity by strains of Kabanoanatake hyphae, A to E, change in protein content, change in color (500 nm), when culture was conducted at the ideal temperature for cultures of around 25°C, on the 110th day of liquid culture (lignin substances as wood constituents were not added to the culture medium). This relationship is an important disclosure which has not been disclosed in the prior technique and gives effective guidance for the harvest of culture medium and antiviral effect, particularly anti-HIV effect.

For example, given the numerical values recorded above for strain B, the active ingredient (anti-HIV agent) can be obtained when additional culture strains are harvested.

Fig. 23 shows a time-course change in the protein content (mg/ml) when 0.8 g, 1.0 g or 1.5 g of lignosulfonic acid sodium salt acetate and lignosulfonic acid sodium salt were added to about 2 l of culture in a 5-l culture container in the liquid culture of Kabanoanatake called as AIW-4R.

Fig. 24 shows a time-course change in the protein content (mg/ml) when 0.8 g, 1.0 g or 1.5 g of lignosulfonic acid sodium salt acetate and lignosulfonic acid sodium salt were added to about 2 l of culture in a 5-l culture container in the liquid culture of Kabanoanatake called as AIW-3R and 58-1.

Fig. 25 shows the suppression of cancer promotion by Compound 1 observed in an experiment using a two-step carcinogenesis model with mouse skin. The promotion time, in weeks, after the application of TBA and the compound in question to the skin is

given on the abscissa. The number of papilloma observed per individual is given on the ordinate.

Fig. 26 shows the suppression of cancer promotion by Compound 1 observed in an experiment using a two-step carcinogenesis model with mouse skin. The promotion time, in weeks, after the application of TBA and the compound in question to the skin is given on the abscissa. The frequency of papilloma occurrence is given as a percentage on the ordinate.

Detailed description of the preferred embodiment

The description of the primary processed matter and the present invention concerning the antiviral therapeutic agents with antibacterial activity is given below; in regard to (I) embodiment as anti-HIV agents, (II) embodiment as anti-bacterial agents, and furthermore (III) embodiment fully as a liquid culture method.

(I) Embodiment as anti-HIV agents

It was found that anti-HIV effects of the extracts from natural Kabanoanatake (primary processed matter) are more potent in the ones derived from the black part on the surface of sclerotia than in the ones from the non-black part (brown) of the internal part, which could prevent, at even 35 ng, the cell fusion (which causes syncytium formation) induced by HIV-infected MOLT-4 cells. In the results of analysis conducted in the same manner regarding the extracts (primary processed matter) obtained by sawdust culture aimed for mass production of Kabanoanatake, it was also seen that 60 to 70% of extracts (primary processed matter) from natural Kabanoanatake have anti-HIV effects, under certain conditions. Furthermore, as a result of the analysis of hyphae grown in liquid culture and dried by heating (dried at 105°C) and a cultured filtrate in the primary processed matter, using HIV-BRU (100TCID₅₀) used PHA-stimulated normal human peripheral blood mononuclear cells, significant anti-HIV effects were revealed in the hyphae grown in liquid culture and in that dried by heating. ED50 (50% inhibition activity) in the inhibition of the syncytium formation of the said natural Kabanoanatake was 35 ng/ml (equivalent to 0.01 µl/ml). These facts show that Kabanoanatake has anti-HIV activity at very low concentration, which cannot be attained by the mushrooms of the two published examples cited above. Remarkably, the activity was found in a state where the active ingredients are not isolated. Moreover, the present invention containing the active ingredients with a molecular weight of less than 450,000 is more active than the components described previously. It

also found that Kabanoanatake extracts of the primary processed matter and the present invention coupled with ingredients of herbal medicine work synergistically, whereby the effects of anti-HIV agents are enhanced and established.

In the present invention, by using natural Kabanoanatake, the extracts obtained from sawdust-cultured Kabanoanatake, hyphae cultured in liquid and dried by heating, cultured viable hyphae, and a cultured filtrate, anti-HIV activity was examined in respect to two forms of the so-called growth of the HIV; the syncytium formation and infection. Detailed account of which is given below. The present invention with a molecular weight of less than 450,000 was more effective than the primary processed matter.

Test samples

- 1 . The black part of Kabanoanatake (natural)
- 2 . Extracts of cultured Kabanoanatake (sawdust culture)
- 3 . Hyphae of cultured Kabanoanatake dried by heating (liquid culture)
- 4 . Viable hyphae of cultured Kabanoanatake (liquid culture)
- 5 . A filtrate of cultured Kabanoanatake (liquid culture)

(1) A test regarding the inhibition of syncytium formation (Fusion Assay) (Fig 1)

When Molt 4 /c18 cells (non-infected cells) and Molt 4 /HIV IIIB cells (infected cells) are cultured together (co-culture) at the proportion rate of 1 : 1, then non-infected cells adhere to the infected cells, which causes cell fusion and syncytium formation, one form of the growth of HIV. When the black part of natural Kabanoanatake (sample 1, the primary processed matter) and extracts of cultured Kabanoanatake (sawdust culture, sample 2, primary processed matter) were added to this co-culture system, a suppressive effect on syncytium formation was observed.

And when the present invention was added, a very high inhibitory effect on the formation of giant cells was observed.

Test procedure

Molt 4 /c 18cells (1×10^6) plus Molt 4 /HIVIIIB cells ($\times 10^6$), the said sample 1 and sample 2 were placed in each well of 96-well microplates respectively, in increments of 100, 10, 1, 0.1 $\mu\text{l/ml}$, and incubated for 24 hours. After that, the diameters of the cells thereof were measured by a multilizer. The cells with diameters greater than $20\mu\text{m}$ were regarded as huge cells, and the appearance rates thereof were measured. The results are

shown in the graphs of Fig.1 and Fig. 1b, with the inhibition ratio for syncytium formation (%) to control on the ordinate and concentration of the samples ($\mu\text{l/ml}$) on the abscissa. Fig. 1 concerns the primary processed matter, and Fig. 1b concerns the present invention. The black part of Kabanoanatake (primary processed matter) demonstrated an inhibition ratio more than twice as strong as AZT. The extracts of cultured Kabanoanatake (primary processed matter) at the concentration of more than $10 \mu\text{l/ml}$ also showed an inhibition ratio that was superior to AZT. Furthermore, it is striking that in the graph of Fig.1 the black part of natural Kabanoanatake of the primary processed matter showed efficient effects assessed as ED50, at the concentration of $0.01 \mu\text{l/ml}$ (equivalent to 35 ng/ml).

The efficacy of the present invention is higher than that described previously, and since the black part of Kabanoanatake achieved ED90 at a concentration of $0.01 \mu\text{l/ml}$ (equivalent to 35 ng/ml) and complete inhibition at $1 \mu\text{g/ml}$, an excellent efficacy of the present invention is observed. In addition, the extract of Kabanoanatake culture also achieved complete inhibition at $1 \mu\text{g/ml}$.

(2) Neutralization Assay (Fig.2 and Fig. 2b)

The other form of the growth of HIV is the so-called infection where a virus jumps out of one cell and gets into a healthy cell. It was examined whether the active ingredients of the present invention could inhibit the infection, with the use of the said samples 1 to 5.

Test procedure

After pretreated stimulated with PHA-blast (PHA [phytohemagglutinin] - stimulated normal human peripheral blood mononuclear cells [PBMC]) (3×10^6) and samples 1 to 5, at the concentration of 100, 10, 1, 0.1, $0.01 \mu\text{l/ml}$ and at the temperature of 37°C for 1 hour, HIV-BRU (0.03 cpm/cell) was added, incubated for 24 hours, washed and further incubated for 5 days. After cell viability was identified and the HIV P24 antigen was measured by ELISA method, the infection inhibition efficacy was studied comparatively. The results were given in the graph of Fig.2 and Fig. 2b. The graph shows the inhibition ratio of infection (ordinate, %), and the concentration of the samples $\mu\text{l/ml}$ (abscissa). High inhibition ratio was observed in the extracts of the black part of natural Kabanoanatake (black circle), particularly it was 70% to 90% at the concentration of more than $1 \mu\text{l/ml}$ (3500 ng/ml). The cultured extracts (white circle) was obtained by sawdust culture. Hyphae cultured and dried by heating (black quadrangle), cultured viable hyphae (white triangle), a cultured filtrate (black triangle)

are all hyphae obtained by liquid culture method, and hyphae dried by heating indicates the hyphae cultured in liquid and dried by heating at 105°C (which smells spicier than the hyphae obtained by other dry techniques) and cultured viable cells indicates the hyphae liquid cultured and then freeze-dried after being boiled in hot water. Hyphae dried by heating (black quadrangle) showed a comparatively high inhibition ratio at the concentration of 0.1 µl/ml.

Next, it was determined how Kabanoanatake affects the cell viability. The two graphs in Fig.3 and Fig. 3b provide the results of the determination. The graph of HIV (-) expresses the results in the non-infectious systems and the graph of HIV (+) expresses the results in the infectious systems. The graph shows the viability (ordinate, %) and test period (abscissa). In the test where Kabanoanatake extracts were used at the concentrations of 0, 3.5, 35, 350, 3500 µg/ml, the amount of living cells was very low at the concentration of 3500 µg/ml, in both the infectious systems and the non-infectious systems. Fig. 4 and Fig. 4b show the HIV P24 antigen yield showed in Kabanoanatake extracts at the same concentration respectively (ordinate, yield concentration of HIV antigen P24 [pg/ml]; abscissa, elapsed number of days), though the low HIV P24 antigen yield is not due to the direct anti-HIV effects of Kabanoanatake, but due to killing the infected or non-infected cells. Turning to the graph of Fig.3 and Fig. 3b, the number of living cells showed in Kabanoanatake was found to decrease at the concentration of 350 µg/ml (black quadrangle) as compared to the control, but there was a difference between the infectious systems and the non-infectious systems. In other words, the number of living cells showed the tendency to decrease in the infectious systems with time, but to recover in the non-infectious systems after the 4th day. This suggests the possibility of Kabanoanatake extracts to specifically kill the infected cells at the concentration of 350µg/ml and to contribute to the activity of phagocyte in the non-infectious systems. The number of living cells as well as viability showed in Kabanoanatake extracts at the concentration of 35 µg/ml, 3.5 µg/ml were almost the same as that of control, and as is shown in the graph of Fig. 4 and Fig. 4b, the HIV P24 antigen yield was well suppressed. This may result from the anti-HIV effects of Kabanoanatake. In this context, the present invention is particularly better than the processed matter.

Next, in the present invention, a test was run for examining whether Kabanoanatake shows anti-HIV effects or not by means of direct action against HIV. The graphs A, B of Fig. 5 show the results of this test. First, Kabanoanatake and the virus were pretreated for two hours, then ultra-centrifuged at 45000 rpm for 90 minutes

to remove Kabanoanatake. The virus was infected with PHA (phytohemagglutinin) - stimulated normal human peripheral blood mononuclear cells (PBMC). As the graph A illustrates the results, it had an almost similar infectivity as the control virus (ordinate, the concentration of the HIV P24 antigen pg/ml; abscissa, the concentration). The point is that the anti-HIV effects of the Kabanoanatake were considered not to affect the virus directly. Next, pretreatment of Kabanoanatake and PHA-stimulated PBMC for two hours, were infected. As illustrated in the graph B, the infection was inhibited depending on concentration at the concentration whereby the viability was relatively maintained in the previous test.

Furthermore, infection inhibition efficacy was determined in the cases where PHA-stimulated PBMC was pretreated with Kabanoanatake for 24 hours, one hour, or not pretreated at all. The graphs of Fig. 6A and Fig. 6A2 illustrates the results. The procedure concerning the graph A and Fig. 6A2 that 100TCID₅₀ of the HIV virus was infected in the presence of Kabanoanatake after pretreatment, washed and then incubated by a culture solution containing Kabanoanatake to measure the HIV P24 antigen released on the 5th day (ordinate, inhibition ratio, %; abscissa, the pretreatment time for target cells by Kabanoanatake). As a result of that, even in the case pretreated for one hour, activity greater than 70 % was observed (90% for the present invention), as was also true in the case pretreated for 24 hours. Furthermore, it was observed that 50 % of the infection inhibition was observed in the case that was not pretreated. In the graphs B and B2 of Fig. 6, the infection inhibition effects of Kabanoanatake on the virus were shown, where Kabanoanatake was added to PBMC pretreated for one hour and infected with the virus in the same manner, on the first, the second and the third day after infection. As a result of that, it appeared that when Kabanoanatake was added to PBMC at a later date, the infection inhibition effects decreased more or less, although even in a culture solution, which does not contain Kabanoanatake, infection was inhibited by 50%. For reasons mentioned above, the active ingredients of Kabanoanatake of the invention may be considered to exert anti-HIV effects when an infection starts. It was also clarified that the pretreatment was more capable of inhibiting infection. Thus, some action of Kabanoanatake on the side of the cells is presumed, and it was found while proceeding in the research that it inhibits reverse transcription and protease.

Fig. 7 and Fig. 7b show the suppressive effect on the syncytium formation by anti-HIV agents of the present invention, when Molt 4/c 18 cells (non-infected cells) are

cultured together with Molt 4 /HIV IIIB cells (infected cells). Correspondently, Kabanoanatake appeared to reduce the syncytium formation depending on the concentration. Kabanoanatake was considered to deeply relate to viral entry steps in HIV. Fig. 7b shows that formation of giant cells is inhibited 100% compared with 80% shown in Fig. 7 at the concentration of 10^{-2} .

As indicated in the next Fig.9 and Fig. 9b (suppressive effect on the syncytium formation), the present invention established culture conditions whereby a strain similar to that of natural Kabanoanatake can be obtained, as the results of attempting to culture Kabanoanatake under various culture conditions, with the object of producing on a large scale an active factor having the anti-HIV effects of Kabanoanatake. Among these, using, in particular, extracts obtained by sawdust culture (white circle) and liquid culture (black quadrangle), as well as the filtrates (white triangle), the suppressive effect on the syncytium formation was determined. The results are given in Fig.9 and Fig. 9b, along with the result in the case of the black part of natural Kabanoanatake (black circle). The graph of Fig. 9 and Fig. 9b shows the results involving the suppressive effect on the syncytium formation by various anti-HIV agents of the primary processed matter (Fig. 9) and the present invention (Fig. 9b), when Molt 4/c18 cells (non-infected cells) were cultured together with Molt 4 /HIV IIIB (infected cells). The ordinate indicates the inhibition ratio (%), and the abscissa indicates the concentration of the anti-HIV agents. Consequently, the five kinds of anti-HIV agents (the black part of Kabanoanatake, sawdust culture, hyphae cultured in liquid and dried by heating, filtrates, cultured hyphae) inhibited the syncytium formation, depending on the concentration. ED50 was observed as to natural Kabanoanatake (black circle) in the extremely small amount of 35 ng/ml ($0.001\text{ }\mu\text{g/ml}$), the inhibition ratio increasing dramatically with the concentration. The extracts obtained by culture appeared to inhibit 50% or more of syncytium formation, as well. Furthermore, filtrates (white triangle) appeared to have inhibition effects against 40% of syncytium formation. The natural product (black circle) in the present invention, on the contrary, has already achieved ED70 at a trace amount of about 35 ng/ml ($0.001\text{ }\mu\text{g/ml}$) and complete inhibition at $1\text{ }\mu\text{g/ml}$. The inhibition rate increased exponentially with concentration. It was found that the culture medium obtained by incubation inhibits the formation of 100% of giant cells. It was, moreover, found that the filtrate (white triangle) shows 70% inhibition.

As has been discussed, sawdust-cultured Kabanoanatake and liquid-cultured Kabanoanatake in addition to natural Kabanoanatake of the primary processed matter

possess anti-HIV effects. The graphs of Fig. 8 and Fig. 8b contain the more detailed information on the test of infection inhibition efficacy. (The ordinate indicates the inhibition ratio (%), the abscissa indicates of the concentration of the anti-HIV agents.) The test of infection inhibition efficacy regarding five kinds of anti-HIV agents was performed at various concentrations as is shown in Fig.8 and Fig. 8b, by means of the samples used in the said Fusion assay. As a result of that, it was recognized that there exist inhibition activity against infection in correlation with inhibition activity against the syncytium formation. Even in the case of the extracts obtained by culture, inhibition activity against infection was observed to a considerable extent, although it is lower than that of natural Kabanoanatake (black circle). In particular, the black part of Kabanoanatake showed an inhibition ratio of 50% at low concentrations, which was not expected previously, for instance, even at the concentration of approximately 0.01 $\mu\text{g/ml}$ (Fig. 8, the primary processed matter). It revealed more potent activity in far lower concentrations than the activity concentration shown in *Lentinus edodes*, *Ganoderma lucidum*, *Flammulina velutipes*, *Auricularia*, etc. described above in relation to the published examples. In comparison with the primary processed matter, the present invention achieved about 80% inhibition at the concentration of about 0.01 $\mu\text{g/ml}$ and 100% inhibition at a concentration of 1 $\mu\text{g/ml}$.

It was recognized that the active ingredients extracted from Kabanoanatake which concerned with the present invention are extremely more effective anti-HIV agents than the primary processed matter, and the efficacy thereof is able to be practically enhanced when the active ingredients are used in combination with the medicinal properties of Chinese herbal medicines. For example, the medicinal properties of Chinese herbal medicines such as *Lithospermum erythrorhizon* (roots), *Cinnamomum sieboldi*, *Prunus persica*, *Pinellia ternate*, *Poria cocos*, *Aconitum japonicum* Thunb (manufactured goods), *Daucus carota* (leaves), *Glycyrrhiza uralensis*, *Schisandra chinensis*, *Zingiber officinalis*, *Asarum sieboldii*, *Prunus armeniaca* and *Rheum palmatum*, etc. used as a mixture or concomitantly with some other agents may be combined or coupled with the present invention, and in fact the combined use of *Lithospermum erythrorhizon* (roots), *Cinnamomum sieboldi*, *Prunus persica* with the active ingredients extracted from Kabanoanatake has been shown to be particularly effective in HIV inhibition when used in combination. It is known that other bacillary virus infection of human cells activates the latent HIV virus, which acts as a trigger for HIV to start to grow and develop symptoms. In order to suppress the activation of HIV occurred by such mixed infection, in particular, the combined or concomitant application of one or more ingredients of

Chinese herbal medicines including *Lithospermum erythrorhizon*, *Cinnamomum sieboldi*, *Prunus persica*, etc. with the active ingredients of Kabanoanatake may protect the internal organs such as liver, kidney of the persons who take them from bacillary virus, and it may also prevent HIV from developing by purifying and strengthening the organs, whereby the active ingredients of Kabanoanatake can reach its potential.

As a practical matter, the primary processed matter concerned with the present invention is able to be extracted by various solvents or various extraction methods. For instance, hyphae of natural Kabanoanatake or cultured Kabanoanatake were treated with PBS solution (3percent) , butanol, ethyl acetate or acetone to give the active ingredients from each insoluble matter.

The active ingredients thereof may be taken out of each non-adsorbent by adsorbing and treating the components or extracts from hyphae of natural Kabanoanatake or cultured Kabanoanatake by carbon or charcoal. Besides, the active ingredients may be extracted even by boiling natural Kabanoanatake or cultured Kabanoanatake in hot water at various pH levels (e.g. for 60 minutes). The sclerotia or hyphae of Kabanoanatake shall be treated with one ore more substances selected from chloroform, ethanol, water, ethyl acetate to obtain the extract from a mixed solution thereof.

The extracted active ingredients obtained by the present invention were recognized to be stable substances which are soluble in water, thermoduric and acid resistant and in any case show inhibition ability against the syncytium formation by HIV and infection inhibition efficacy at an almost equal level. Kabanoanatake which is used in the present invention include, in addition to the said sawdust-cultured or liquid-cultured Kabanoanatake, hyphae and its secretions proliferated artificially by planting Kabanoanatake hyphae on the green wood of birch trees, or that which was made to form sclerotia artificially by planting Kabanoanatake hyphae on the green wood of birch trees. These Kabanoanatake produced by artificial planting of hyphae are closer to natural products than cultures, accordingly included in natural Kabanoanatake in the present invention.

The effects on immunity improvement in an HIV patient who has taken the present invention orally are described. After the active ingredients of Kabanoanatake of the primary processed matter were given by mouth to the HIV-positive patient, in an early stage (60 days after administration), in the middle stage (150 days after administration), immunity code was measured by L.F.T. (Life Field Tester) in the later stage (547days after administration). The measurement was done by analyzing the patient's first urine of the morning, using L.F.T. The immunity code was -7 on the 60th day, approximately 0

on the 150th day, and the improved figure, +7 was obtained on the 547th day. The figure -7 is said to be at the same level as lower figures of normal persons in magnetic field analysis. In addition, the mean immunity codes in normal persons are around +13. The active ingredients of Kabanoanatake of the present invention was also proved to be effective by blood examination, as is shown in the figure, from where it is recognized that they may be effective preventive agents as well as therapeutic agents. For the present invention, on the contrary, the immune code was determined with L.F.T. (life field analyzer) in HIV-infected patients with similar symptoms in the early phase (60 days after administration), the intermediate phase (150 days after administration) and the later phase (547 days after administration). For determination, the first morning urine of the patient was collected and subjected to L.F.T. The immune code was -7 on 60 days but increased to 0 on 80 days and improved to +14 on 547 days. The figure of +14 can be said to be similar degree of immunity to healthy subjects in the analysis of magnetic field. The present invention was found to be more useful than the primary processed matter.

(II) Embodiment as anti-bacterial agents

In the present invention, the anti-bacterial activity of Kabanoanatake extracts (the primary processed matter and the present invention) was tested and confirmed regarding pathogenic bacteria, in particular, the various bacteria listed below in Table 1 and Table 1a, that is to say, *Escherichia coli* O157, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Clostridium perfringens*, according to the minimum inhibitory concentration method (MIC) (Japanese Society of Chemotherapy). Aside from *Clostridium perfringens*, each seed fungi of a specified amount was inoculated into a medium for sensitivity disks (the numbers of inoculated bacteria, Table 1), and *Clostridium perfringens* was tested by being inoculated into Brain-Heart agar culture medium with 0.1% sodium thioglycollate added, using the method described.

One way to produce Kabanoanatake extracts using anti-bacterial activity test in the present invention is as follows. In the first example, 100 g of powdered Kabanoanatake (natural or artificial cultures), for instance, is placed in 1000 cc of distilled water, and extracted by hot water at the temperature of 100°C for 40 minutes. In the second place, the extract was filtrated through gauze or paper filter, and was centrifuged. In the second example, the extract was filtrated through gauze or paper filter, and was centrifuged. It was centrifuged, for example, by a separator at 3000 rpm for 10 minutes.

Furthermore, it was filtrated again and freeze-dried to obtain the active ingredients of Kabanoanatake extract (the primary processed matter) aimed at. It is subjected to gel filtration to obtain the present invention with the molecular weight of less than 450,000. The effects of this anti-bacterial activity test are given in Table 1.

Table 1 (antibacterial effects of the primary processed matter)

Pathogenic bacteria	Numbers of inoculated strains	MIC (ppm)
MRSA (*) (methicillin resistant <i>Staphylococcus aureus</i>)		5,000
<i>E. coli</i> O-157(EDL 931)	2.7×10^7	10,000
<i>K. pneumonise</i> (ATCC 4352)	8.0×10^6	10,000
<i>P. aeruginosa</i> (IFO 13276)	2.8×10^6	5,000
<i>S. aureus</i> (IFO 12732)	2.2×10^7	5,000
<i>B. subtilis</i> spore (ATCC 6633)	1.4×10^6	100,000
<i>C. perfringes</i> H2	2.0×10^6	2,600

(*) After being cultured on SOD medium at 37°C for a night, methicillin resistant *Staphylococcus aureus* was prepared to 10^6 CFV/ml, incubated into Mueller-Hinton agar medium containing the test specimen (Kabanoanatake extract) in a two-fold dilution series, using Micro-Planter, and cultured at 37°C for 18 hours, to give minimum inhibitory concentration.

Table 1a (antibacterial effects of the present invention)

Pathogenic bacteria	Numbers of inoculated strains	MIC (ppm)
MRSA (*) (methicillin resistant <i>Staphylococcus aureus</i>)		2,500
<i>E. coli</i> O-157(EDL 931)	2.7×10^7	4,700
<i>K. pneumonise</i> (ATCC 4352)	8.0×10^6	5,000
<i>P. aeruginosa</i> (IFO 13276)	2.8×10^6	4,700
<i>S. aureus</i> (IFO 12732)	2.2×10^7	2,500
<i>B. subtilis</i> spore (ATCC 6633)	1.4×10^6	47,000
<i>C. perfringes</i> H2	2.0×10^6	1,100

(*) After cultured on SOD medium at 37°C for a night, methicillin resistant *Staphylococcus aureus* was prepared to 10^6 CFV/ml, incubated into Mueller-Hinton agar medium containing test specimen (Kabanoanatake extract) in a two-fold dilution series, using Micro-Planter, and cultured at 37°C for 18 hours, to give minimum inhibitory concentration.

To ascertain as to whether the anti-bacterial activity is a peculiar property to Kabanoanatake or other mushrooms have similar anti-bacterial action, the following test was performed. Candidate mushrooms included *Granolas frondosa* (Dicks:Fr), *Lentinus edodes* (Berk) Sing. in addition to Kabanoanatake. 100 g of raw materials of these candidate mushrooms were placed in a vessel, respectively, with 1000 cc of distilled water, and were boiled at 100°C for 40 minutes to obtain extracts. By filtrating these extracts through Toyo filter paper No. A5, 200 cc extracted test liquid was obtained from each candidate mushroom. Anti-bacterial test was run as regards these extractives, by means of medium for sensitivity disks, through the method, similar to the one described above. The results are shown in Table 2 and Table 2a.

Table 2

Samples	Types of bacteria	Numbers of incubated stains	MIC
(1)	<i>E.coli</i> O157(EDL 931)	2.7×10^7	10,000 ppm
(2)	<i>E.coli</i> O157(EDL 931)	1.0×10^6	not hampered
(3)	<i>E.coli</i> O157(EDL 931)	1.0×10^6	not hampered

Sample (1)=Kabanoanatake extracts boiled by hot water (primary processed matter)

Sample (2)=*Grifola frondosa*

Sample (3)=*Lentinus edodes*

Table 2a

Samples	Types of bacteria	Numbers of incubated stains	MIC
(1)	<i>E.coli</i> O157(EDL 931)	2.7×10^7	5,000 ppm
(2)	<i>E.coli</i> O157(EDL 931)	1.0×10^6	not hampered
(3)	<i>E.coli</i> O157(EDL 931)	1.0×10^6	not hampered

Sample (1)=Kabanoanatake extracts boiled by hot water (primary processed matter)

Sample (2)=*Grifola frondosa*

Sample (3)=*Lentinus edodes*

Anti-bacterial activity tests of Kabanoanatké cultures were performed concerning helicobacter pylori. The results appeared to be effective. The procedures are as follows.

1. Samples

As samples, four kinds of the present invention, natural Kabanoanatké extracted with hot water (primary processed matter), cultured Kabanoanatké extracted with hot water (primary processed matter) and liginosulfonic acid sodium salt, were used.

2. Adjustment of samples

Each sample was extracted using DMSO at 10 w/v % to make stock solution, further from which ten-fold serial dilution was made by DMSO, to prepare the test solution.

3. Helicobacter pylori culture and adjustment of strain suspension

H. pylori. strain NCTC 11637 and strain NCTC 11916 were cultured on blood agar which added 5 % defibrinated horse blood to Brucella Agar (BBL) at 37°C for 3 days, under microaerophile surroundings (N₂ 80%, CO₂ 15%, O₂ 5%). The strains were suspended in Brucella broth added 0.5mg/ml of BSA fraction V (Sigma A-4503) to a concentration of 10⁸ cfu/ml, to prepare inoculum organism liquid, and 0.1 ml of the liquid was smeared on BSA agar added the said BSA, after that anti-bacterial activity against *H. pylori* was determined by Disc method, as described below.

4. Disc diffusion method

Each test solution of 20 µl was made to absorb in a sensitivity disk (8 mm in diameter, Advantech, thin-model), placed on agar smeared with strains, cultured under microaerophile surroundings at 37°C for 3 to 5 days. After that, growth-inhibitory zone around the disk showing more than 1 mm was regarded as positive.

5. Results

The results are as follows.

Table 3 The results of a test involving antibacterial activity of each sample, using extracted DMSO stock solution.

		Antibacterial activity: +, presence ; -, absence	
		<u>H. pylori strain number</u>	
Samples		11637	11916
1	Cultured Kabanoanatake	+	+
2	Natural Kabanoanatake	-	-
3	Lignosulfonic acid sodium salt	-	-
4	Present invention	+	+
Control (reference)			
antibiotics	test method	MIC (µg/ml)	
		11637	11916
		blood agar	BSA agar
		blood agar	BSA agar

Erythromycin Disk *	6.3	3.1	6.3	1.6
agar plate dilution method	0.05	0.03	0.05	0.03

*Growth-inhibitory zone around a disk showing more than 1 mm is regarded as minimum concentration (Minimum Inhibitory Concentration)

Although the basis of evidence that the present invention has anti-bacterial properties, which are not seen in other mushrooms, is pseudohumic acid (polyphenol complex), especially regarding the biological activity for keeping human bodies from bacteria known as immunostimulating effects. Cells concerning immunology may be lymph corpuscle and macrophage, LPS (lipopolysaccharide) contributing to one of the macrophage activities. The test was conducted in order to ascertain how much of the constitution of the Kabanoanatake is recognized to be important by the present inventor, the results are given in the Table 4. As samples, carrot extract and carrot leaf extract were used in addition to Kabanoanatake. To prepare the samples, separate portions of 500 g of Kabanoanatake, carrot extract and carrot leaf extract were weighed and distilled water for injection was added to them to a final concentration of 100mg/ml. These were extracted by heating at 56°C for three hours in hot water, and then centrifuged, from which the supernatant was taken to make into the stock solution for measuring samples. LPS was determined by colorimetry, Limulus amoebocytelysate assay (Seikagaku Corporation) to measure LPS specifically.

Table 4

Samples	LPS content (μg/g)
Kabanoanatake extract (primary processed matter)	1.601
Kabanoanatake extract (present invention)	1.813
Carrot extract	0.85
Carrot leaf extract	29.5

(III) Embodiment of liquid culture method

(1) Practical incubation

According to the principals of the present invention, the details of the practical liquid culture method are described hereafter. Although incubation vessels are not limited to the specified ones, 5L Erlenmeyer flasks are suitable for convenience in handling and ease of manageability. The following is the example of the standard medium culture composition (liquid).

malt extract (Baltimore Biological Laboratory) 20 g
 glucose 20 g
 peptone (Nihon Seiyaku) 6 g
 yeast extract (Oriental Yeast, Co., Ltd.) 6 g
 1/100M potassium phosphate buffer (pH5.0) 200 ml
 water (tap water, ground water or snow water) 1800 ml

Thus, 2 liters of standard liquid medium can be made. They all are placed into a 5L Erlenmeyer flask to make a liquid medium. When incubation vessels are larger or smaller than this, a liquid medium may be prepared in proportion to the said combination ratio. The flask containing liquid medium is closed with the silicon stopper loosely placed on the flask. Part of the silicon stopper is covered with aluminum foil. The medium flask should be autoclaved at 121°C for 20 minutes for sterilization by heating. After the flask is cooled, the silicon stopper is sealed tightly, which should be done on a clean bench, to make doubly sure. A large incubation volume may be possible using many medium flasks at the same time. The said Kabanoanatake hyphae incubated before test is added to these medium flasks, and then incubated. Actually, various strains are used, but hereunder in Table 5, the several values regarding three selected typical bacterial strains, A-2, A-6, A-7 are shown. Furthermore, in these cases, the fluid volume at the initiation of incubation was 200 ml because the test was conducted in a 500-ml Sakaguchi flask.

Table 5

Kabanoanatake strains	Culture days	pH (liquid)	Protein mg/ml	Glucose mg/ml	Brix %	500nm	Final liquid measure (114 th day)
A-2	28	7.26	0.135	0.093	0.8	1.4323	
	70	8.46	0.2338		1.2	1.390	
	114	8.04	0.416	0.162	1.1	1.8802	140ml (70%)
A-6	28	5.41	0.058	0.093	1.3	0.5187	
	70	8.15	0.2610		1.0	1.694	
	114	7.92	0.404	0.058	1.0	1.8592	140ml (70%)
A-7	28	6.22	0.227	0.140	1.3	2.1426	
	70	8.15	0.4093		1.0	1.751	
	114	8.04	0.494	0.100	1.1	1.9272	142ml (72%)

Anti-HIV virus activity was measured by the test method using the said 96 well microplate regarding these three kinds of bacterial strains on the 28th, 70th, 114th day.

The effects are described below along with other findings. 100 % inhibition activity against HIV was examined in a two-fold dilution series.

A-2: 500 nm absorbance was 1.4323 on the 28th day of culture, which was a good beginning. It decreased somewhat to 1.390 on the 70th day, but increased to 1.8802 on the 114th day of culture. In the meantime, the effective dosage for anti-HIV activity was 250 (on the 28th day), 250 (on the 70th day) and 62.5 µl/ml (on the 114th day).

A-6: Due to its low colored secretions, 500 nm absorbance was 0.5187 on the 28th day, which was not a good beginning. 500 nm absorbance increased to 1.694 on the 70th day, and increased further to 1.8592 on the 114th day. The effective dosages for anti-HIV activity during the periods were zero (on the 28th day), 125 (on the 70th day) and 62.5 µl/ml (on the 114th day).

A-7: 500 nm absorbance increased dramatically to 2.1426 on the 28th day of culture. Afterwards, it was lowered to 1.751 on the 70th day, which increased again to 1.9272 on the 114th day. The effective dosages for anti-HIV activity were 125, 31.25 and 125 µl/ml.

On the whole, in the liquid culture method of the present invention, black color showing the active ingredients appears on approximately between the 25th to the 33rd day. This indicates that the active ingredients may be obtained in a relative short period of time. However, there are some incubated areas where the production of the active ingredients are low, which is improvable by adopting measures such as interfusion of lignin substances as wood constituents (described later according to the present invention) into a culture medium, addition of humic acid, or increase in the amount of culture medium. Among these techniques, however, since it was found by research that active ingredients can be produced steadily by addition of lignin substances (wood substances) to the culture medium and solid medium (if necessary) in resistance to the culture temperature significantly inhibiting the growth of the hyphae (decreased from 10°C to 8°C) and even if the growth is stopped due to incorporation of oxygen for a certain period (11 hours a day), it was disclosed in this application. Thereafter, the extract of hyphae obtained by culture and the culture medium or solid artificial culture (culture in sawdust) are extracted by a given method and fractionated by molecular sieve (gel filtration), and the active ingredients of this application can be obtained by collecting the fractions with a molecular weight of less than 450,000. Different from the prior techniques in literatures in which no active ingredient develops even if live hyphae are present, steady production of active ingredients was realized for the first time in the world after long efforts and disclosed in this application.

Concerning doses

As concerns the actual dose, the daily dose is 0.50 to 1.80 g if the present invention of the Kabanoanatake extract with a molecular weight of less than 450,000 is used. It shall be, for example, divided into 3 or 4 doses and taken before meals. It shall be taken after dissolution in water or lukewarm water. It is preferable to use lukewarm water. For a patient weighing about 60 kg, for example, the daily dose of the present invention is 0.9 g to 1.8 g. In the 3 divided doses, 0.3 g to 0.6 g of the present invention shall be taken at 7:00 a.m. before breakfast. Breakfast shall be taken 30 minutes after. In the second administration at 11:30 a.m., 0.3 g to 0.6 g of the present invention shall be taken. Lunch shall be taken at about 12:00 p.m. In the third administration of the present invention, 0.3 g to 0.6 g shall be taken at 5:00 p.m. This is a basic method of administration in a day. It is not prohibited to undergo a fourth administration. At such the time, the present invention shall be added as described previously. After the third administration, the fourth administration shall be conducted at 10:00 p.m., and good result may be obtained by administration of 0.3 g to 0.6 g.

It is known that, since HIV (Human Immunodeficiency Virus)-infected patients have weakened immunity, they frequently develop various diseases such as cancers and caposi sarcoma. Generally, anticancer agents are administered for cancers, and antibiotics are administered for *Helicobacter* bacteria. Therefore, multiple adverse reactions appear. It is an actual condition that various disorders such as vertigo and gastrointestinal disorders occur. The present invention contains pseudo-humic acid and triterpene, and has bioactive effects. The present invention was developed for the purposes of eliminating or reducing the above-mentioned disadvantages of multiple-drug treatment, eliminating the drug-resistance of retrovirus and inhibiting the related bacterial diseases while retaining the quality of life of virus-infected patients. The present invention with a molecular weight of less than 450,000 is of course easily absorbed compared with common mushrooms; the daily dose of the extract for the purpose of suppressing HIV viruses of *Lentinus edodes*, for example, is given as 9 g. The dose of the present invention is much lower than that, and in comparison with the primary processed matter of Kabanoanatake, it is very effective at much lower doses.

Concerning anti-cancer activity

In addition to the previously mentioned Kaposi's sarcoma, skin cancer, bladder cancer, prostate cancer, and a variety of other cancers occur with unfortunate frequency.

Therefore, research was undertaken in order to obtain valid scientific data supporting the suppression of cancer. As a result, a substance, triterpene, in the present invention with molecular weight less than 450,000 was found to have anti-cancer properties. Chloroform extraction is preferable for triterpene, so the resulting substance could, for example, be intentionally mixed with the present invention with molecular weight below 450,000 obtained from hot water extraction in order to increase the concentration. Of course, it is also possible to add additional triterpene compounds, either singularly or in any combination, to the present invention. Naturally, the present invention could be dosed together with triterpene in order to increase the effects or to supplement them with the additional anti-syndrome effects of the present invention. In addition to oral administration, injection and intravenous drips are possible. The present invention and other comparable substances were compared with triterpene used in these ways. The results of an anti-cancer experiment showing the suppression of carcinogenetic promotion by the active ingredients together with the triterpene compounds shown in chemical structure formula (Claim 27), or their pharmaceutically acceptable salts, are disclosed below.

In recent years, the two-stage carcinogenesis model has become widely accepted. In this model, the development of cancer caused by chemical substances (chemical carcinogenesis) is divided into the stages of initiation and promotion. Initiation describes the process in which a chemical agent, the “initiator,” irreversibly damaged the DNA of a normal cell and changes that cell into an “initiated cell.” Promotion refers to the process in which a chemical agent, the “promoter,” causes the proliferation and progression of the “initiated cell,” eventually becoming a cancerous cell. If both processes could be restrained, the development of cancer would be suppressed. In particular, although it is not possible for an “initiated cell” to return to normalcy, suppression of carcinogenetic promotion is a valid means of carcinogenetic suppression. The inventor discovered the anti-cancer effects, that is, the suppression of carcinogenetic promotion (also referred to below as promotion suppression effects), of the triterpene compounds extracted from the sclerotia of Kabanoanatake.

Namely:

1. The triterpene compounds shown in chemical structure formula (Claim 27), or their pharmaceutically acceptable salts.
2. The process described in Claim 27 for acquiring triterpene compounds or their pharmaceutically acceptable salts from the sclerotia of Kabanoanatake constitutes a method for producing triterpene compounds.

Although asymmetric carbon is present among the compounds of the present invention, all of the compounds present are configured in a nearly optimal way for promotion suppression effects to occur. Chloroform extraction was established as shown in Figure 7. The extraction efficiency was approximately 1000 times better than that of ethanol extraction. Methanol extraction was performed after chloroform. The triterpene fraction is not eluted in the methanol extract. In addition, experimental proof, using mice, was given for the first time regarding triterpene's in-vitro inhibition of EBV-EA and in-vivo suppression of carcinogenetic promotion.

The following method was used to measure the carcinogenetic promotion suppression effects of the chemical compounds of the present invention. EBV-EA induction assaying [reference: Konishi, T. et al., *Biol. Pharm. Bull.*, 21, 993 (1998)] can be given as an explanation. Carcinogenetic promoters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) and teleocidin, and Burkitt's lymphoma human lymphoblastic cells with a dormant Epstein-Barr virus (EBV) infection (Raji cells, available from ATCC), were used in this assay. The activation of the EBV virus dormant in the Raji cells was used as a basis for measurements. Specifically, the appropriate process used was to first add carcinogenetic promoters such as the above, together with the substance in question, to the Raji cells and measure the substance's suppression of EBV activation caused by the promoter. The majority of compounds tested exhibited a high level of correlation between in-vivo suppression of EBV activation and in-vitro suppression of carcinogenetic promotion.

Finally, it is possible to evaluate in-vitro carcinogenetic promotion effects using rodents and the two-stage carcinogenesis experiment. For example, the hair on the back of a mouse could be cut using surgical scissors, and 7,12-dimethylbenz-anthracene (DMBA), dissolved in an organic solvent such as acetone or methanol, soaked into the skin in an appropriate concentration, for example, on the order of 1 μ g/1000 μ g. After a suitable period of time has elapsed (for example, one week), the substance in question could then be applied to the skin at an appropriate interval (for example, twice per week). More specifically, the substance in question could be dissolved in an organic solvent such as the above and then soaked into the skin where DMBA was applied. After an appropriate period of time (for example, one hour), TPA dissolved in an organic solvent such as the above at an appropriate concentration (for example, 0.1 μ g/10 μ g) could be applied to the same skin. While proceeding with this treatment for a suitable period (for example, 10-50 weeks), chronological observation of the skin could be performed, and the number of tumors that appear on the treated skin, or the number of mice that develop such tumors, could be counted. Then, it would be possible

to determine the effectiveness of carcinogenetic promotion suppression of the substance by comparing the number of tumors, or the number of mice that developed tumors, in the group where the substance and TPA were applied with the control group where only solvent was applied.

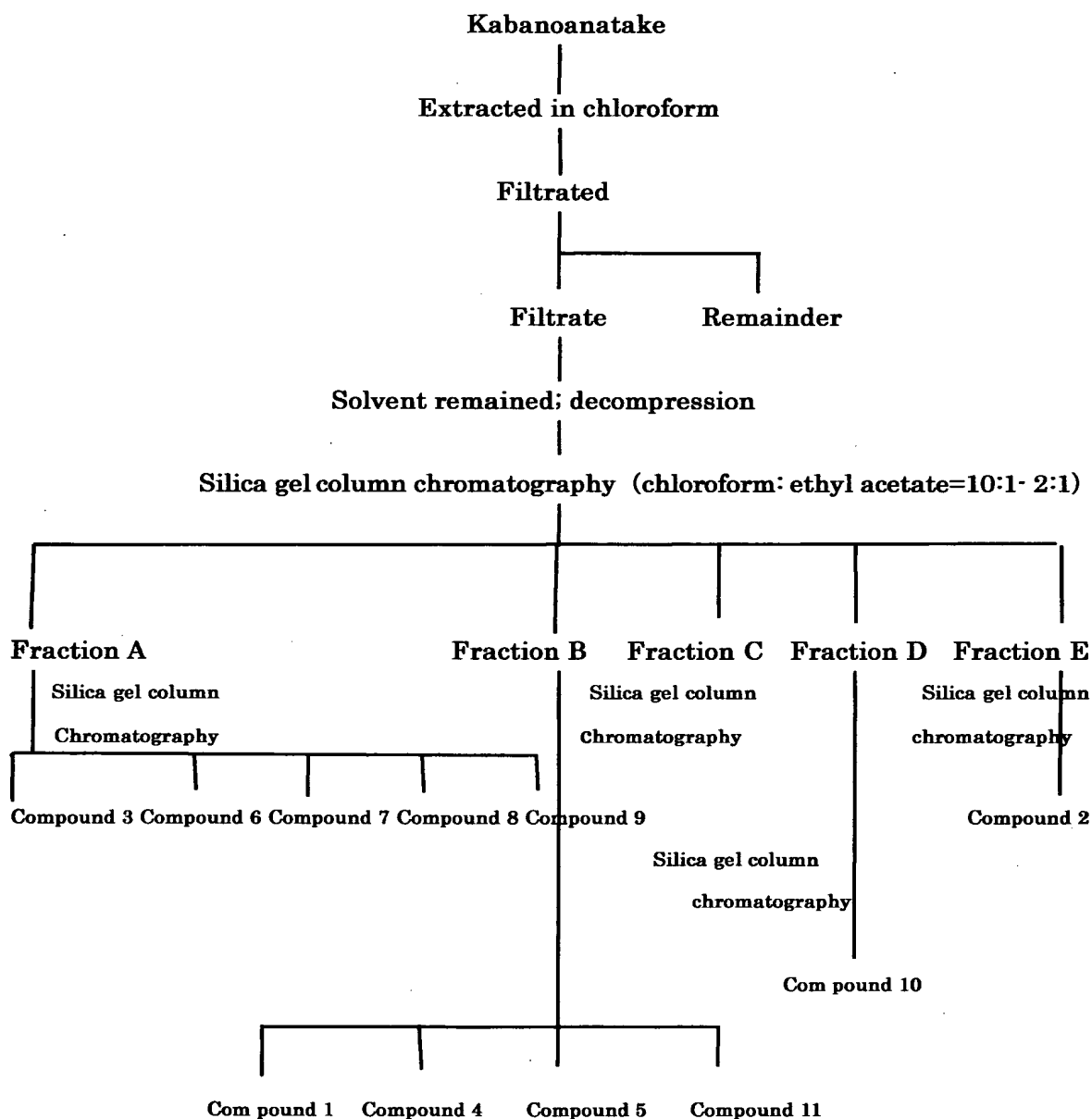
[Example of Execution]

Here the current invention is again explained in detail using an example of execution. However, it is not limited in any way to what is expressed here.

Reference 1 (Preparation of the compound)

As shown in Table 6, the entire quantity of the appropriate extract was dissolved in chloroform and then column chromatography was performed using 3.5kg silica gel prepared in chloroform (Merck Silica Gel 60). A solvent mixture of chloroform and ethyl acetate (chloroform:ethyl acetate=10:1) was used and fractionation was performed on each 1L. Fraction A was obtained from collecting fractions 40-46 (18.0g, 7x1L) and Fraction B from collecting fractions 47-52 (49.0g, 6x1L). Next, the chloroform:ethyl acetate mixture was adjusted to 5:1 and fractionation was performed on each 1L. Fraction C was obtained from collecting fractions 62-70 (3.0g, 9x1L) and Fraction D from collecting fractions 71-79 (6.0g, 9x1L). Fraction E was obtained from collecting fractions 80-105 (12.5g, 26x1L). Each fraction obtained above was then purified using silica gel column chromatography or high speed liquid chromatography (HPLC) using ODS and methanol. Thus, the chemical compounds were isolated. By performing silica gel column chromatography on Fraction A, Compound 3 was obtained from the fraction eluted in chloroform, Compounds 6 and 7 from the fraction eluted in chloroform:ethyl acetate=5:1, and Compounds 8 and 9 from the fraction eluted in chloroform:ethyl acetate=2:1. In Fraction B, Compounds 1, 4, 5 and 11 were obtained from the fraction eluted in chloroform. In Fraction D, Compound 10 was obtained from the fraction eluted in chloroform. In Fraction E, Compound 2 was obtained from the fraction eluted in chloroform:ethyl acetate=5:1.

Table 6



Trial 1 (Measurement of Promotion Suppression Effects)

The promotion suppression effects of Compounds 1, 2, 2-acetate, 3, 4, 5, 6, 7, 7-acetate, and 11, obtained in Reference 1, were determined using the EBV activation suppression assay (Konishi, T. et al., Biol. Pharm. Bull., 21, 993 (1998)). PPMI1640 culture medium (Nissui Pharmaceuticals), with cow embryonic serum (GIBCO-BRL) added to

arrive at 10v/10v%, was used to culture Burkitt's lymphoma human lymphoblastic cells with a dormant Epstein-Barr virus (EBV) infection (Raji cells). The activation rate of EBV in this culture medium (the natural activation rate of Raji cells) was less than 0.1%. The culture fluid for the Raji cells cultured in the medium described above was prepared to achieve a concentration of 1×10^6 cells/ml, and a solution of butyric acid (final concentration 4mM) in DMSO, as well as TPA (final concentration 4ng/ml), was added. This was placed in a CO₂ incubator for 48 hours at 37°C and a smear preparation of the resulting culture fluid was prepared. Using serum acquired from a nasopharyngeal carcinoma patient, EBV early antigens (EBV-EA) were stained using IFA, and the incidence rate of positive cells (cells where early antigens are expressed) was taken as the positivity control (100). On the other hand, a solution of butyric acid (final concentration 4mM) in DMSO, TPA (final concentration 4ng/ml), and the test compound were added to culture fluid for the Raji cells prepared in the above-mentioned manner, and the result incubated in the same way. The incidence rate of positive cells was then determined and calculated as a percentage based on the control. At least 500 cells were counted in each experiment, and each was repeated 3 times. Furthermore, a negative control, where only butyric acid, and neither TPA nor the test compound, was added, was also undertaken in parallel each time, in addition to the above-mentioned positive control.

The results are presented in Table 7. There was a correlation between dose size and suppression of EBV-EA activation in every compound tested. In addition, none of Compounds 1, 2, 2-acetate, 3, 4, 5, 6, 7, 7-acetate or 11 exhibited strong toxicity in the cells in any of the experiments.

Table 7

Test Substance	Incident Rate of Positive Cells ¹⁾			
	Concentration of Test Substance (moles compared to TPA)			
	1000	500	100	10 ²⁾
Compound 1	2.4 (70)	37.6	66.1	93.1
Compound 2	9.2 (70)	45.0	75.4	98.6
Compound 2-acetate	12.7 (70)	47.8	79	100
Compound 3	4.9 (70)	44.7	74.3	96.3
Compound 4	3.2 (70)	40.3	69.5	95.9
Compound 5	0 (70)	35.1	64.0	90.4
Compound 6	8.9 (70)	44.1	73.8	95.1
Compound 7	6.3 (70)	42.0	71.3	94.3
Compound 7-acetate	7.9 (70)	47.0	78.1	96.7
Compound 11	12.1 (70)	49.2	79.1	100

1) The value of the positive control (with TPA) was set as 100.

2) The value in parentheses denotes the cell survival rate (positive control set at 100%)

Promotion suppression effects were determined based on a comparison of the number of mice that developed papillomas, and also the average number of tumors per mouse, between the group treated with test compounds and the group treated only with solvent. The results are shown in Figures 25 and 26, where the suppression effects of Compound 1 on carcinogenetic promotion by TPA are evident.

Additionally, a dose per body weight of 1000mg/kg was found to be safe in mice for each ingredient.

Trial 2 (Suppression of carcinogenetic promotion on mouse skin)

Carcinogenetic promotion suppression effects were examined regarding the Compound 1 obtained in Reference 1, using the two-stage carcinogenesis model and mouse skin.

The hair on the back of a 6 week old ICR female mouse was shaved and a solution of 0.1 ml acetone and 100 mg (390 nmol) 7.12- dimethylbenz[a]anthracene (DMBA) was applied. A solution of 0.1 ml acetone and Compound 1 was applied to the same site in the experimental group one week after the application of DMBA. At the same time, 0.1 ml acetone was applied to the solvent (control) group. A solution of 0.1 ml acetone and 1 mg (1.7 nmol) of TPA was applied to the site in both groups one hour later. TPA was applied to both groups in this manner twice per week for 20 weeks. Each group consisted of 3 mice and the experiment was repeated three times. Promotion suppression effects were determined based on a comparison of the number of mice that developed tumors, and also the average number of tumors per mouse, between the group treated with test compounds and the group treated only with solvent. The results are shown in Figures 25 and 26, where the suppression effects of Compound 1 on carcinogenetic promotion by TPA are evident.

Study of growth inhibition effects on P388 mouse lymphocytic leukemia cells

Methods:

P388 mouse lymphocytic leukemia cells were cultured at 37°C using eagle Minimum Essential Medium including 10% cow embryonic serum. The culture fluid was then centrifuged at 900 rpm for 5 minutes when the concentration reached approximately 7×10^5 cells/ml. The supernatant was discarded and a fixed amount of culture medium added to the remaining cells to produce a cell suspension of 1×10^5

cells/ml (initial concentration). On the other hand, the specimen was placed in solution with DMSO (10mg / ml) and then diluted with culture medium to reach 200,20,2µg / ml. The control solution was diluted so that the concentration of DMSO was 2,0.2,0.02%. 100 µl of the cell suspension and either the test solution or the control solution was placed in each hole of a 96 hole microplate. The result was then cultured in a CO₂ incubator at 37°C for 3 days. Next, MTT was added (6 mg/ml, phosphate-buffered fluid) and the cells dyed. Finally, 50µl of sodium dodecyl sulfate was added (20%, 0.02NHCl), the result placed in solution with formazone, and set aside for one night. The absorbance was then measured in a microplate reader (BIORADmodel450).

The following equation was used to calculate the growth (G) percentage, using a semi logarithmic graph and the ED₅₀ concentration where 50% of cell propagation is suppressed.

$$G \% = \frac{\text{sample absorbance} - \text{initial concentration absorbance}}{\text{control absorbance} - \text{initial concentration absorbance}} \times 100$$

Results

The results are given in Figure 8 below. A gentle inhibition of cell proliferation was observed in three of four compounds (1, 5, 7) tested, with the exception of Compound 2.

Table 8

Test Compound	50 % growth inhibition concentration ED ₅₀ (g/ml)
1	30.4
2	>10
5	27.0
7	22.0

Concerning anti-influenza virus activity

A preclinical study of the primary processed matter and the present invention concerning the influenza virus is presented below.

Virus studied: Influenza type-B (type B/Bangkok) (amount of virus: 100 TCID)

Test solution: 20 mg of the present invention and the primary processed matter were each added to 10 ml of distilled water, producing 0.2% solutions.

Cells: MDCK (test-tube cultured) were used.

Observation period: 5 days 0.2 ml of test solution and virus were combined at 37°C with 1.0 ml of liquid culture fluid (preservation), (effective concentration $0.2 / (0.2 + 0.2 + 1.0) = 0.1428571$ ml/ml, weight per volume 285.7 µg/ml.) This was placed in the first of twelve wells, then two-fold serial dilution was carried out in each of the following wells, and CPE (cell damage) was observed daily. It was observed that CPE was suppressed through the third well (4-fold dilution), and so the minimum effective concentration (MIC) was 71.4 µg/ml (weight per volume). In this way, it was confirmed that Kabanoanatake extracts (the present invention) effectively suppress the influenza virus. It was also observed in the same manner as above that the present invention was effective against Influenza type B/Hokkaido, type A/Hong Kong and type A/Hokkaido (8-fold dilution). The results for the present invention are shown in Table 9, while the results for the primary processed matter are shown in Table 10.

Table 9 Anti-influenza effects of Kabanoanatake extract (the present invention)

Virus strain	B/Bangkok	B/Hokkaido	A/Hong Kong	A/Hokkaido
	(B)	(B)	(H3N2)	(H1N1)
MIC (µ/ml)	71.4	71.4	35.7	35.7

Table 10 Anti-influenza effects of Kabanoanatake extract (primary processed matter)

Virus strain	B/Bangkok	B/Hokkaido	A/Hong Kong	A/Hokkaido
	(B)	(B)	(H3N2)	(H1N1)
MIC (µ/ml)	142.8	142.8	71.4	71.4

* Looking at the minimum effective concentrations (µg/ml), the increased effectiveness of the present invention, extracted from Kabanoanatake, over the primary processed matter was observed.

It is known that in influenza infections, the virus enters host cells and, via the processes of transcription and reproduction, new viruses are formed and leave the host cell. During this time, the enzyme neuraminidase removes the sialic acid receptors on the virus, enabling it to leave the host cell and propagate. It is believed that the present invention inhibits the activity of the enzyme neuraminidase. After mixing the present invention with the influenza virus in a container, the degree of migration to mouse

pneumocyte was examined under microscope. It was found that migration to the lungs was highly inhibited. Multiple, uniform results of this kind showing effectiveness against the influenza virus have never been previously published. Therefore, this constitutes a strong hint of the exceptional degree of anti-viral activity of the present invention.

In addition to the influenza virus, effectiveness against the infectious agents *Streptococcus mutans* and *Streptococcus sobrinus*, members of the cavity-inducing *mutans streptococci*, was observed.

Cavities in infants are particularly common from 9 to 36 (31) months after birth, the so-called “window of infectivity.” These cavity-causing bacteria mainly enter the infant’s mouth from the mother, although cases of infection from pets are also known.

Concerning vancomycin resistant enterococci and methicillin-resistant *staphylococcus aureus* (MRSA)

If the anti-methicillin-resistant *staphylococcus aureus* effects exhibited by Kabanoanatake are examined, the active ingredients responsible are identified as linolenic acid and linoleic acid. Additionally, the same linolenic acid and linoleic acid are the active ingredients responsible for the anti-vancomycin resistant enterococci (VRE) effects. These exist in the present invention with molecular weight below 450,000, and so the antibacterial action of the present invention is explained below based on the extracted active ingredients.

A minimum inhibition concentration (MIC) study was conducted to evaluate antibacterial effectiveness against vancomycin resistant enterococci (VRE) and methicillin-resistant *staphylococcus aureus* (MRSA) using standard plate culture agar.

Methods:

An appropriate amount of each specimen was placed in a culture medium for use with sensitivity disks and agar plates were prepared. 25 ml of each bacterial strain under study (prepared at 10^6 / 1 ml) was placed on the plate and then cultured for 48 hours at 37°C. Each strain was then checked to determine if growth had occurred.

The minimum inhibition concentrations (MIC) for vancomycin resistant enterococci (VRE) and methicillin-resistant *staphylococcus aureus* (MRSA) were then identified. A variety of resistant strains acquired from hospitals and 20 preserved strains

were used. The results are shown in Table 1

Table 11

	VRE	MRSA
linoleic acid	0.0156 – 0.125 (%)	0.0078 – 0.0156 (%)
linolenic acid	0.0313 – 0.0625 (%)	0.0078 – 0.0313 (%)

- Each experiment was performed on 20 antibiotic resistant strains obtained from Japanese domestic hospitals.

In these experiments, the MIC found for vancomycin resistant enterococci (VRE) and methicillin-resistant staphylococcus aureus (MRSA) is recorded in Table 11. The antibacterial activity was found to be between 0.0156 – 0.125 and 0.0078 – 0.0156 percent. (Staphylococcus aureus was 0.0078 percent). The MIC of linolenic acid was 0.0313 – 0.0625% for VRE and 0.0078 – 0.0313% for MRSA. The inventor is the first person to discover the inhibition effects of linoleic and linolenic acid on VRE and MRSA. Possible practical applications include: doses of a medicine containing the active ingredients, or application to sheets or underwear to prevent infection on the skin.

According to the CDC's National Nosocomial Infections Surveillance (NNIS) system, the rate of VRE infections that occurred in U.S. hospitals rose from 0.3% to 7.9% between 1989 and 1993. Although it is a result of this increase, the rate of VRE infections for ICU patients increased an alarming 34-fold. The rate of VRE infections in NNIS-registered hospitals was connected to the size of the hospital (200 beds or more) and university affiliations. Examples of prevailing infections and of sporadic infections in other hospitals were also published. Because the automated method in use at most laboratories is unable to identify VCM-resistant strains, and in particular moderately resistant strains (shown in VanB phenotype), the actual incidence rate of VRE infections in American hospitals is possibly even higher. The frequency of VRE strains that are highly resistant to medications such as penicillin and aminoglycoside is likely to make the challenge of treating the infectious diseases caused by such bacteria even more difficult. Choices of treatment for such cases are limited, and both experimental drugs and antibacterial agents without firm studies of their effectiveness are in use.

The epidemiology of vancomycin resistant enterococci (VRE) is still unclear, but certain patient groups can be considered to be at high-risk for VRE infections. These groups include: patients with extremely severe illnesses, patients with serious diseases and patients with depressed immune systems (for example, patients in the ICU, tumor ward or organ transplant ward). In addition, the following patient groups can also be

considered at high-risk for VRE infections: patients who have undergone heart or abdominal surgery, those who have urethral or nutritional catheters, long-term patients, or those receiving a particularly large variety of antibiotics or vancomycin. Enterococci normally reside in the alimentary canal or female genitalia, and these, normally present, bacteria can cause infections in patients. However, recent research indicates that most infections are caused either by direct transmission between patients, or by indirect transmission, through the medium of medical staff, or contaminated surfaces and medical equipment. Anxiety over such infections is currently rising in countries with advanced medical care. In particular, VRE infections are troubling, as the use of penicillin increases the frequency of antibiotic-resistance, often leaving patients with little medical recourse. In this context, the present invention provides a much-needed combination of anti-VRE, anti-MRSA and pseudo-humic acid properties. In patient groups such as the above-mentioned, or in the treatment of infectious bacterial diseases, the present invention can play a crucial and fundamental role by combating both VRE and MRSA infections. The potential role of the present invention as an antiviral drug endowed with preventive and therapeutic effects concerning infectious bacterial diseases is critically important, in both AIDS and general wards.

SOD-like action

Next, the present invention's SOD-like action is examined. SOD (superoxide dismutase) acts inside of organisms such as bacterium and mammals to protect the organism from the toxicity of oxygen radicals. The present invention acts in a similar manner, referred to herein as "SOD-like action." Using special equipment, it is possible to measure the level of this activity.

Measuring device: JEOL model JES-RE-1X ESR (electron spin resonance) device

Test sample: Kabanoanatake added to the below-mentioned purified water at a concentration of 50 mg / 50 ml.

Purified water: Japanese pharmacopeia purified water.

Result: SOD-like activity was measured as 11.56 (Units/ml), thus remarkable SOD-like activity was observed.

Remarkably high SOD-like activity was also observed when tap-water (Musashino-shi, Tokyo) was used. Similar activity was also observed when melted snow-water (a pure water with fine clusters made by melting new snow from Nayoro-shi, Hokkaido at low temperature) was used. The results of the same experiment using the primary processed matter were 8.55 (Units/ml). To quickly summarize the situation: because a

higher than required level of oxygen radicals in the body causes harm to all patients, a medicine with SOD-like activity protecting the body from this toxicity is currently desired by society. The SOD-like activity of the present invention was more than 3 units higher than that of the primary processed matter. Additionally, the results of this SOD-like activity, and its potential usefulness in, for example, treatment of the pancreas during diabetes, protection and repair of internal organs, and atopic dermatitis, is worthy of attention by the world's academic conferences. Furthermore, the extremely high SOD-like activity compared to other foodstuffs, in combination with the other effects (antiviral, antibacterial) of the present invention, provides for a wonderful therapeutic power with strong and varied pharmacological effects, extremely limited side effects, and ability to retain quality of life. A simple example is that of atopic dermatitis. If 1.5 g is divided into three daily doses (morning, afternoon and evening), an incremental improvement will generally be noticed by the 10th day. The patient usually recovers after roughly 3 months of this treatment. (Note that creams containing the present invention are also effective for skin patients.) Quality of life includes the cases of increased physical strength (grip strength increased from 2 to 5 kg/F), and, due to stimulation of the cerebral cortex, is shown in the increased energy and boldness following doses of the present invention reported in the personal experiences of a clinical pathologist. Leg strength, swallowing ability, excretion strength, and mental vigor increase following doses of the present invention. Thus, the present invention combines a high level of SOD-like activity with the ability to aid in the recovery of physical and mental power. It is an antiviral agent with therapeutic effects for syndromes that increase the quality of life in affected patients.

Concerning anti-fat agglutination effects

Presently, society is continuing to shift from manpower to machine-power, and meals have become increasingly high in calories. Fat has become an increasingly important problem in this social background, with excess fat accumulating in the body leading to increases in obesity, fatty livers, appearances of cerebral hemorrhage and diabetes. Therefore, a study of the effect of the present invention on body fat was undertaken.

Excess fat in domesticated chickens often clings to the anus, and can prevent the discharge of eggs or excrement. This occurrence was utilized in the experiment.

Methods:

40 30-day old domestic chickens (20 white-feather, 20 red-feather) were used. In the control group (untreated), 30cc of water and a fine white feed powder (available from

Hokuren) was placed in the feeding tray for each bird following feeding. The experimental group was given a solution of the present invention and 30cc of water, together with feed powder, in the same manner. Dosage was set at the equivalent of 1.5-3g / 60kg body weight and administered once per day. The chickens were raised in this way for 2 years, 8 months. The chickens were then dissected and the amount of fat clinging to internal organs was determined.

Results:

In 9% of the control group (untreated), fat accumulated along the liver and anus and prevented the adequate discharge of excrement, leading to a buildup of excrement in the body, which caused the dissolution of half of the liver. 41% of the control group showed an abnormal accumulation of fat in the subcutaneous region, liver, gizzard and anus. 0% of the experimental group (both the 1.5g and 3.0g/60kg body weight groups) showed an excessive degree of fat in the anal region. Fat in the subcutaneous region, liver and gizzard was observed to a small degree. The color of the liver rose to 90% in the experimental group, with 9% being particularly beautiful and vivid. The results indicated that the present invention helps prevent the abnormal buildup of fat near the liver, potentially retarding the aging of this vital organ. A similar result was observed in the mouse experiments, with a general reduction of body weight noted in the experimental group. These are not side effects. Beneficial slimming of human patients has also been observed.

The above results indicate that the present invention could be useful in treating illnesses, especially diabetes, caused by obesity and the unhealthy accumulation of fat near internal organs. Following administration of the present invention (1.5 g / day divided into 3 doses) to a 55 year-old male type-2 diabetes patient, after 30 days his blood sugar level had fallen and he became eligible to enroll in an insurance program. A similar case of a 60 year-old female patient had been receiving insulin injections for 30 years. She suffered from a feeling of physical tiredness and heaviness. Her hemoglobin A1C was 9.0, but after administration of the present invention (1.5 g / day divided into 3 doses) for 30 days, it fell to 7.9 and her feeling of heaviness were relieved. Hand dynamometer measurements immediately following administration of the present invention rose to 34 kg / F compared to 30 kg / F when she was untreated. This patient had frequently developed benign polyps in the past (the development of polyps in the colon and elsewhere is generally inevitable in diabetes patients), but an examination following 6 months of continual doses of the present invention showed her blood condition to be satisfactory and an endoscopic examination found no polyps in the colon. Furthermore,

her weight fell from 65 kg to 61 kg.

Cases where the present invention appeared to help liver damage also exist. A 48 year-old male with acute cirrhosis of the liver showed a favorable improvement after 46 days of continuous administration of the present invention. A male with Hepatitis-B showed a favorable improvement after 40 days. Dosage in both cases was 1.5 g / day, divided into 3 doses taken in the morning, afternoon and evening. Their respective average grip strengths increased from 50.5 kg / F to 53.0 kg / F and from 42.5 kg / F to 45.2 kg / F.

The present invention is effective against a variety of related diseases. The results concerning fat are clear from the standpoint of clinical pathology, and the above-mentioned cases can be considered representative of the therapeutic effects of the present invention.

Concerning Kabanoanatake's active ingredients

As mentioned above, the active ingredient largely responsible for Kabannoanatake's antiviral effects is a pseudo-humic acid based on ordinary methoxyl. If even more similar lignins are examined, it is found that combination with sodium gives lignin sulfonic acid antiviral effects, including anti-influenza effects, at a lower concentration than lignin sulfonic acid alone. The respective MICs for lignin sulfonic acid against influenza type-H1N1, type-H3H2 and type-B were 1.9 mg/ml, 1.9 mg/ml and 2.5 mg/ml. A lignin sulfonic acid sodium compound was approximately 20 stronger, with respective MICs of 0.16 mg/ml, 0.01 mg/ml and 0.66 mg/ml. Additionally, the MIC of lignin sulfonic acid for 100% suppression of HIV propagation was 15.6 µg/ml, while that of the lignin sulfonic acid sodium compound was 3.91, roughly 4 times more powerful. Humic acid contains a very small amount of methoxyl-based substances and they themselves exhibited no anti-AIDS effect in an experiment. However, using the standard 2-fold serial dilution test, the MIC of humic acid Na for 100% suppression of HIV (100TCID) was found to be 0.01 µg/ml, a truly amazing result. Cytotoxicity was low and these results are certainly useful. Antibacterial effects were also observed. Pseudo-humic acid is largely responsible for the antibacterial and antiviral effects of the sodium-rich Kabanoanatake. In particular, the crucial components were found to be pseudo-humic acid Na and humic-acid Na compounds. A chemical analysis of humic acid Na is shown in Table 12 for further reference.

Table 12

Na ₂ O	7.4723%
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Al ₂ O ₃	0.4172%
SiO ₂	1.1802%
SO ₃	2.5823%
K ₂ O	0.1317%
CaO	1.9987%
TiO ₂	0.0820%
V ₂ O ₅	0.0031%
MnO	0.0110%
Fe ₂ O ₃	0.8701%
SrO	0.0132%
C ₆ H ₁₀ O ₅	85.2383%

Main example of the methods of treatment

For example, for the treatment of infection with AIDS virus, the virus in the blood shall be decreased to about 0 as determined by the ELISA method and the tissue culture infection dose (TCID) test after administration of this drug, and Kabanoanatake shall be cultured as it is by method 1 and the virus shall be reduced drastically. Thereafter, 3 other antiviral drugs approved and marketed as drugs shall be mixed by method 2, and consequently, a good result can be achieved. Actually, a male patient (50 years old) infected with AIDS virus who acquired AZT resistance only by taking AZT drugs for 3 months eliminated infection with AIDS virus by the method 2 by taking this drug for 1.5 years and was able to recover healthy body functions. The efficacy of this drug appears earlier than the primary processed matter of Kabanoanatake.

It should be said to be an epoch-making therapy utilizing this drug. Or, when a 58-year old male patient with adult T-cell leukemia (ATL) took this drug at a daily dose of 0.1 to 0.5 g in 3 divided doses in the morning, in the afternoon and at night, complexion was improved and sinking feeling disappeared in 6 months, and he became able to do daily work energetically.

When a 60-year old female patient infected with the hepatitis B virus took this drug at a daily dose of 0.1 to 0.5 g in 3 divided doses in the morning, in the afternoon and at night, her lung-power increased and sinking feeling disappeared, and she became able to do light work in 3 months and daily work in 6 months. In a 53-year old male patient, complexion was improved and sinking feeling disappeared, and he became possible to do daily work in 3 months.

A 57-year old businessman infected with the influenza virus had a fever and suddenly collapsed into bed. When 0.3 g of the present invention was dissolved in water and taken twice every 6 hours, the fever was eliminated, and he could return to his work. It was found that the present invention not only shows antiviral effects but also

increases the grip strength from 3 to 5 KgF immediately after administration. It is one of the epoch-making effects unique to the present invention, which is not observed in any anti-HIV agent distributed recently.

Since the present invention acts repressively on the syndromes associated with AIDS infection, if the present invention is inserted into the vagina or the anus, it is desirable to insert a daily dose of 0.5 to 1.8 g at one time. If a person is assaulted by a thug, and if the thug is a carrier of the HIV virus, since the present invention directly inhibits viruses in its presence in the surrounding tissues even if HIV virus is released into the vagina or the anus, the present invention prevent invasion of the virus into cells. As described in the specification, in a discrimination test in which the body fluid collected by adsorption onto the gauze was diluted with water, it was found to be effective in the discrimination test that the anti-HIV effect did not decrease even if the present invention was incorporated into the vaginal fluid after administration and even after exposure to the conditions in the vagina for 72 hours. Therefore, the viral infection rate in the human body may be decreased by preventing viral infection in the local area due to physical assaults.

In addition, as concerns the use of the present invention with a molecular weight of less than 450,000 in the anus, absorption from the rectum is also expected. The components with anti-AIDS activity begin to diffuse and adhere to the vagina or the anus. By the presence of the active ingredients of the present invention which are finer than the primary processed matter, adherability and absorbability improve. For example, the present invention diffuses in the localized area to prevent infection in the blood vessels, blood and lymph in the anus. In order to avoid excretion of the present invention together with feces, it is desirable to insert the present invention after defecation. If feces are excreted after the use of the present invention, moreover, it is desirable to use the daily dose in the anus again. For insertion, the present invention may be wrapped with a soluble capsule or a sugarcoat film. The present invention is considered to exhibit its antiviral activity and the antibacterial activity described later repressively in the surrounding of the uterus or anus. This method of use is different from that of common vaccines, but the present invention is fine and has antibacterial and antiviral activities, and if the present invention exists in the site at a certain concentration in advance, it can inhibit directly the infection due to invasion of virus and achieve preventive effects. Although it is not applicable to the functions in the whole body as described in the concept of vaccines, it can prevent viral infection in the localized area due to physical assaults.

Analysis method

As common practice, the following method has been adopted. Each sample was counted to an accuracy of 200 mg/ml, and dissolved in deionized water. Stirred thoroughly and centrifuged at 3,000 rpm for 15 minutes, the supernatant solutions thereof were taken and subjected to the following test.

Experiment equipments

spectrophotometer; Shimazu spectrophotometer UV-1200

p H meter ; Toua Digital p H meter-50

A. OD 500 nm and p H

After the preparation of 1% (w/v) aqueous solutions of each sample, the absorbance was read at 500 nm and p H was measured, using a routine method.

B. Protein

The protein content in each sample solution was assayed by the Bradford method. The sample solution, 20 μ l, was placed into a 1.5 ml test tube and was combined with 1 ml of Bradford solution. The mixture was left standing at room temperature for 5 minutes, and then the absorbance was read at 595 nm. As a control group, Bradford solution added to 20 μ l deionized water was employed. The sample solution was diluted ad libitum and measured, being careful not to be affected with impurities contained in the samples. The protein content in each sample solution was calculated, referring to a calibration curve created using bovine serum albumin as a reference, and converted into the amount per 1g sample.

C. Glucose

Using glucose C- II Test Wako (Wako Pure Chemical Industries, Ltd.), the glucose content in each sample solution was measured. The reagent used for this measurement was an enzymatic reagent with high specificity. The sample solution, 20 μ l, was placed into a test tube and was combined with 3.0 ml color-producing reagent. After being warmed to 37°C for 5 minutes, the absorbance of each solution was read at 505 nm. As a control group, sample solution added 3.0 ml of deionized water was employed, thus the color effect was eliminated. Calibration curves were produced by simultaneous reaction to the glucose standard solution. The glucose content in each sample solution was calculated, referring to the calibration curve and converted into the amount per 1 g sample

D. All saccharides

Using a phenol-sulfuric acid method, the total amount of saccharide in each sample was measured. The sample solution, 20 μ l was placed into a test tube and was combined with 5% phenol solution, 200 μ l. Then, 1 ml of concentrated sulfuric acid was added dropwise to the test tube, and stirred rapidly. After being left standing at room temperature for 20 minutes, the absorbance was read at 490 nm. As a control group, distilled water was used instead of the sample solutions. Calibration curves were produced by simultaneous reaction to the glucose standard solution. The sample solution was calculated as the amount of glucose, referring to the calibration curve and converted into the amount per 1 g sample.

(2) Addition of sap of white birch

According to the said liquid culture composition, the effects of the addition of sap of white birch to water were determined. The sap of the white birch was added by 5%, 10%, 40% and 90% in total. They were incubated for 42 days along with the control, to which the said saps were not added, and observed regarding Brix (%), the amount of dried hyphae (g), the quantity of glucose and protein (mg/ml), redox potential (mV), and pH, as indexes. As a result, at the concentrations of more than 5 %, an increasing amount of dried hyphae of Kabanoanatake was seen. Redox potential was also low at the concentration of more than 40%, and the amount of protein increased. Through these results, it was found that the sap of the white birch has a good effect on the liquid culture, by adding at least 40%.

(3) Incubation by snow water

Moisture is essential to the culture solution. To ascertain that the culture results depend on the different quality of water, water from melted snow was used to culture Kabanoanatake. The snow of the Nayoro region in Hokkaido where the inventor is domiciled is of much better quality, compared to that of other regions. Another study of the present inventor proved that it helps plants such as tulips grow better, and improves the skin texture of human beings. We sell this snow water under the trademark of "Yukinohada (skin of snow)". The incubation of hyphae of Kabanoanatake was conducted by means of this snow water (100%) and ground water of the said Nayoro region. The strains used for both are the same. The culture composition is essentially equal to that of the said (3), except for using snow water. The effective dose for perfect inhibition of HIV during a specific time period in which CD₄ cells were

co-cultured with the HIV virus was investigated in a two-fold dilution series. It was determined to be more than 62.5 $\mu\text{l/ml}$ for the samples cultured in snow water and to be more than 500 $\mu\text{l/ml}$ for the controls (ground water) on the 3rd day. The former was determined to be more than 125 $\mu\text{l/ml}$, and the latter more than 500 $\mu\text{l/ml}$ on the 7th day. From this fact, secretions from the hyphae cultured in snow water was seen to have as much as four times the activity of that cultured in ground water. With respect to cell damage of both samples, the former was confirmed to be 250 $\mu\text{l/ml}$, the latter to be 1000 $\mu\text{l/ml}$, whereby the safety of cultured Kabanoanatake is assured.

(4) Addition of wood constituents (lignin substances, betulinic acid, etc.)

In the said liquid medium method of Kabanoanatake, in particular, fluctuation of the incubation room temperature under the influence of outdoor air temperature in winter (33°C to 8°C), etc. may cause growth delay in some hyphae, which may be a causative of melting and killing hyphae as the days pass. If it occurs prior to the secretion of the active ingredients, it may be troublesome in cultures. The inventor has also researched in earnest, as the subject matter, a way to initiate anti-HIV activity in ideal incubation conditions more stably in a short period of time. Thorough the investigation, addition of lignin substances, etc. contained in wood constituents as the major constituents into the said liquid medium revealed to energize hyphae of Kabanoanatake, without reducing its vigor, and to help anti-HIV effects thereof to be more stable.

In regards to wood constituents, there are lignin sulfonic acid, lignosulfonic acid sodium salt, lignosulfonic acid sodium salt acetate, lignin alkali, lignin organosolv, lignin organosolv acetate, 2-hydroxypropyl ether, lignin hydrolytic, hydroxymethyl derivative, lignin organosolv propionate, betulin (betulinic acid), or lignin salts, etc. In such lignin substances, lignin salts are included. It is advisable that, in liquid cultures, the property of the substances be soluble in water as much as possible, although it is not limited to this property. Betulin becomes soluble when cultured with Kabanoanatake in the culture medium. Among them, lignin sulfonic acid (Kanto Kagaku Corporation) exerted a high degree of effectiveness. In general, the wood constituents are used in the range of concentration between 0.00001% and 0.00075 % (weight) for the said liquid medium. For instance, in the case where lignin substances were used at 0.000293% (weight percent) by preference, anti-HIV effects could be yielded in the initial stage in a short time and high activity had been kept after the days passed. However, in the case where the weight percent was above 0.00075 %, considerable growth suppression was seen in the hyphae on the liquid medium. Hereafter, three types of Kabanoanatake

hyphae are explained, referred to as AIWro-4, A-2W-3, AIW-27, in order to distinguish them for convenience.

(a) In order to determine the perfect anti-HIV effects (100%), a neutralization assay was performed by using Kabanoanatake hyphae (AIW ro-4 hyphae incubated in the liquid medium for 62 hours), co-cultured the said CD₄ cells and the HIV virus. The experiment was conducted to examine whether the HIV virus was inhibited or not in a two-fold dilution series, by incubating Kabanoanatake at 33°C in the daytime and 8°C at night of room temperature and obtaining extracts thereof from the culture vessels. (Since the desired temperature for the growth of Kabanoanatake is approximately 25°C, lowering the incubation temperature is regarded as an extreme condition). In the untreated control test area, lignin substance was not added. In the control area, perfect inhibition of the HIV virus was seen at 125 µl/ml, when the concentration level of HIV was 10 TCID₅₀ on the 3rd day of the neutralization assay when HIV does not increase so much. However, on the 6th day when viral amounts increased, the perfect HIV inhibition was not observed in the area containing 100 TCID₅₀ of virus. On the contrary, in the test area where 0.3g of a lignin substance, lignin sulfonic acid of wood constituents was added to the said liquid medium, which came to a total weight of 2052 g including 2 liters of water (weight percent, 0.000146%), HIV was inhibited perfectly at the concentration of 31.3 µl/ml (10TCID₅₀), and at 62.5µl/ml (in 100 TCID₅₀), where stable inhibition effects and good growth of hyphae was observed. Similarly, in the test area added 0.6g of lignin sulfonic acid to the said medium (weight percent, 0.000292%), perfect inhibition was observed on the 3rd day of the assay at 15.6 µl/ml (in 10 TCID₅₀), and on the 6th day at 62.5 µl/ml (100 TCID₅₀). This test area where 0.6 g lignin sulfonic acid was added was recognized to show higher inhibition than that where 0.3 g lignin sulfonic acid was added, using 10TCID₅₀ of virus, at an early stage of this neutralization assay.

(b) Likewise, A-2 W-3 hyphae of Kabanoanatake (cultured for 34 days in a liquid culture) was investigated by the neutralization assay under extreme conditions. In the test area without adding any lignin sulfonic acid, the perfect inhibition was seen at 250 µl/ml (10 TCID₅₀), but it was not seen when the viral level was 100 TCID₅₀. On the other side, in the area added 0.3g of lignin sulfonic acid, 100% inhibition effect was observed at 250 µl/ml, even using 100 TCID₅₀ of virus. Also in the area added 0.6 g of lignin sulfonic acid, the effects were recognized to increase, at 31.3 µl/ml (10 TCID₅₀), and at 125µl/ml (100TCID₅₀).

(c) The results are described hereafter as regards a long-term culture test under extreme conditions restricting the infiltration of oxygen.

Under extreme conditions of diurnal incubation temperature of 25 to 33°C and night incubation temperature falling to 8°C to 10°C, the effects on liquid culture with lignin substances added were investigated, using the same lignin sulfonic acid together with two kinds of Kabanoanatake hyphae (referred to as AIW-27 and AIW-4 for convenience), on the 36th, 47th, 70th, 78th, and 100th day of culture. Practically a great number of wood constituents such as lignosulfonic acid sodium salt acetate were the subjects of investigation and proved to be effective. In the following, lignosulfonic acid is described on behalf of the wood constituents. Lignosulfonic acid is referred to as lignin hereunder. This test illustrated in Fig.13, Fig.14, Fig.17, Fig.18 and Fig.19 was performed under extreme conditions for Kabanoanatake, of diurnal room temperature at approximately 25 to 33°C, where hyphae are incubated. 2052 g of the said culture medium and O₂ are mixed in a liter Erlenmeyer flask, with reciprocal shaking at 51 cycles/min. Usually reciprocal shaking is carried out continuously for an entire day (24 consecutive hours), though in this investigation it was carried out for only 11 hours and after that shaking was not performed (extreme conditions for cultures). During winter, the incubation room is not warmed at night, so that the minimum temperature thereof becomes 8°C to 10°C, which was utilized for this investigation. As the result of that, in the said liquid medium, two kinds of Kabanoanatake hyphae (AIW-4, AIW-27) to which lignin was not added, were not able to grow under such extreme conditions, and were killed before the 36th day of culture, in which the HIV inhibition was not seen. On the other hand, hyphae in all the test areas survived. Regarding anti-HIV activity, on the 36th day, perfect anti-HIV inhibition was attained at 250 µl/ml, in the test area with AIW-4 and 0.6 g of lignin as well as in the area with AIW-27 and 0.6 g of lignin, whereas it was not observed in either area with 0.3 g of lignin. On the 47th day, in the area with AIW-4 and 0.3g of lignin and in the area with AIW-4 and 0.6 g of lignin, the virus was inhibited at 125 µl/ml. In the area with AIW-27 and 0.6 g of lignin, perfect inhibition was observed at 250 µl/ml. In the area with AIW-27 and 0.3 g of lignin, it was not seen. On the 70th day when the activity dramatically increased, the HIV virus was perfectly inhibited in the area with AIW-27 and 0.6 g lignin and in the area with AIW-4 and 0.3 g lignin, at 15.6 µl/ml, and in the area with AIW-4 and 0.6 g lignin, at 32.3 µl/ml. In the area with AIW-27 and 0.3 g lignin, very high inhibition of the virus was observed at 15.6 µl/ml. Successively, on the 78th day of culture, excellent inhibition activity was shown at 15.8 µl/ml in the area with AIW-27 and 0.6 g of lignin, in the area

with AIW-27 and 0.3 g of lignin and in the area with AIW-4 and 0.6 g of lignin. It was also shown at 62.5 $\mu\text{l/ml}$ in the area with AIW-4 and 0.3 g of lignin. Furthermore on the 100th day of culture, perfect inhibition was achieved in the area with AIW-27 and 0.3 g of lignin and in the area with AIW-4 and 0.6 g of lignin, respectively, at 62.5 $\mu\text{l/ml}$, and also it was achieved at a high level of 32.3 $\mu\text{l/ml}$ in the area with AIW-27 and 0.6 g of lignin and in the area with AIW-4 and 0.3 g of lignin. The present test was run under extreme conditions. Since the culture room is not warmed, the minimum temperature thereof falls to 8°C to 10°C during winter at night. Even under such extreme conditions, when the wood constituents were added, astonishingly the hyphae did not die and were kept alive in all the experimental areas for a prolonged period. As already stated, excellent inhibition effects on HIV (100%) were achieved on the 78th day of culture, in the area with 0.3 g of lignin, and it was also achieved even on 100th day, in the areas with 0.3 g or 0.6 g of lignin. It was revealed that more potent HIV inhibition activity was achieved in the area with 0.6 g of lignin (32.3 $\mu\text{l/ml}$), than in the area with 0.3 g of lignin (62.5 $\mu\text{l/ml}$). Throughout the present invention, the average ratio of solid content of Kabanoanatake hyphae to the liquid culture was 5.31 mg/ml. For instance, when 100CTD₅₀, of virus is used, the effective amount, 15.6 $\mu\text{l/ml}$, is converted into 0.828 $\mu\text{g/ml}$ by solid content, which indicates 100% inhibition of HIV. Other wood constituents have a quickening effect on Kabanoanatake. Birch lignin dissolved by boiling sawdust of white birch and filtrated to be isolated from the sawdust may be added, by calculating the amount of solid content of lignin in the liquid medium. Addition of lignin substances including finely ground lignin and bark of birches as wood constituents to a culture medium was effective in maintaining a stable culture of Kabanoanatake, not only under extreme conditions mentioned above, but also at the ideal temperature for cultures of around 25°C. Differing from Kabanoanatake adhered to the timbers in the forests, Kabanoanatake hyphae obtained by liquid culture appeared to have instability in the quality of survival activity (pharmacological activity). However, it has been recognized that the addition of lignins and betulin (betulinic acid) as wood constituents enables anti-HIV effects to be obtained stably, in addition it strengthens the life force of Kabanoanatake and increases its potential bioactive effects in animals and human beings, such as antimicrobial action, superoxide dismutase activity and anti-mutagenicity. And this technique for utilizing Kabanoanatake is recognized as being useful, in decomposing lignin substances, according to need.

In addition, for the liquid culture of Kabanoanatake, the addition of 0.1g/L to 0.9 g/L of amino acid (such as L-alanine, DL-aspartic acid, glutamic acid, or L-methionine),

or of malt extract, helps to stabilize the production of triptenes, which is useful in increasing the vitality of the hyphae and its bioactive effects on humans and animals.

(5) The effect of light on cultures

The growth level of two types of strains which are dissimilar in character, K-AIW and K-BIW was checked both in cases where they are exposed to the light and those where they are not, regarding pH, glucose content, protein content, 500 nm absorbency (production of secretion of coloring constituents), etc. To quickly summarize the results, Kabanoanatake hyphae were proven to have a different sensitivity to light (availability) depending on the strains. For example, it is better to expose light on K-AIW, where original protein and burnt umber components were well produced, whereas it is rather better not to expose light on K-BIW, where more protein and coloring constituents were developed. Consequently, in ensuring the anti-HIV activity of cultured Kabanoanatake, it is important to consider the involvement of light depending on the growth level of cultured hyphae (whether exposed to light or not), in addition to the index in judging such as coloration level in cultures, quantity of protein in the liquid medium, decrement of carbon source in the liquid medium, pH of the liquid medium, and so on. The condition for culture that allows the rapid absorption of carbon sources, including glucose and other medium nutrients, thereby inducing growth of hyphae by aphotic culture in the initial stage of growth and then exposure to light is confirmed to increase protein content and have higher activity in some strains (ex. K-AIW). In the above test, natural light was used as light (it had not been exposed at night), but artificial light may also be used.

(6) Addition of humic acid

In cultures by standard liquid medium, in practice, anti-HIV activity may sometimes be lower. In order to improve the quality and yield of the cultures, a variety of studies have been conducted, wherein the test by addition of humic acid to liquid medium revealed significant effects. Namely, 0.3 g of humic acid was added to 2 liters of standard liquid medium (wherein commercial reagent of humic acid is available). Anti-HIV effects when this liquid medium was cultured in a 5-liter flask at 25°C in a shake culture are shown hereafter. At 100 TID₅₀, however, no difference was observed. In order to produce the active ingredients more steadily and to increase the efficacy, it was found to be significantly beneficial to add wood substances. The efficacy appeared even at 100 TID₅₀. It can be used for a culture in a jar fermenter. (The details are disclosed in the specification.)

* Effective dose for perfect HIV inhibition on the 3rd day (the amount of virus, 10TCID₅₀), co-cultured CD₄ cells and the HIV virus

<u>Strains</u>	<u>Culture medium</u>	<u>Effective dose</u>	<u>Cell damage inhibition</u>
K-BIW (cultured for 70 days)	without humic acid	above 250 µl/ml	above 250 µl/ml
	addition of humic acid	above 125 µl/ml	above 250 µl/ml

(In this case, 'cell damage' is different from 'cytotoxicity', because constituents of Kabanoanatake are derived from natural objects and are taken orally.)

(7) Modification of liquid medium

It is possible that a liquid medium attenuates in a long-term culture (150 days, reciprocal shaking, 25°C). To improve this, four test areas (A, B, C, D) were prepared beforehand by means of a 5 liters flask respectively, wherein 2 liters of standard medium were placed along with additional components, as follows:

A: Standard medium (without modification)

B: 0.3 g of humic acid was added to standard medium on the 61st day after initiation of culture.

C: On the 76th day after hyphae strains were placed into the standard medium, one half of a medium that has the same composition as standard medium was added (to 1000 cc of water).

D: On the 61st day after incubation was initiated on the standard medium, 0.3 g humic acid was added, and furthermore on the 76th day 0.3 g of humic acid and one half of the standard medium were added.

Anti-HIV activity (100 % inhibition of syncytium formation) in each areas expressed by index was as follows; regarding A as 100, B as more than 200, C as more than 250, D as more than 300. The modified culture medium was found to achieve a preferable high activity, compared to (A), the culture medium which was not modified from the initiation of incubation. Describing it in detail, when the same carbon source was used, (A) showed as much as twice the anti-HIV activity (per culture medium volume), as the test case of humic acid described above. The test area (B) where one half of the carbon source (the same composition as the medium) was added at the time the carbon source was consumed showed as much as two point five times the perfect

inhibition activity for HIV (per culture medium volume) as the standard medium. In the test area (D) where humic acid was combined with additional culture medium, the filtrate, as it is, caused cell damage (which does not indicate 'cytotoxicity') and made it unable to be measured. Consequently it was centrifuged to spin down to a solid content, which showed further high activity as (C) in the supernatant thereof.

(8) Culture in a jar fermenter

As an example of a large-scale liquid culture method, large-scale culture of Kabanoanatake was cultivated using a 30-liter jar fermenter.

For the jar fermenter, MSJ-U2 (30-liter) was used (B. E. Marubishi, Co., Ltd). First, to prepare seed fungi, hyphae grown on PDA (potato dextrose agar) punched with a No.4 cork borer were placed into 300 ml culture solution in three flasks. Here, a 500 ml shaking flask was used to leave more room for volume. The shaker rotated at 100 times/min., it was shake cultured at 25°C for 31 days. The culture medium contained hyphae and was placed on the jar fermenter in an amount equivalent to five shaking flasks. 20 liters of culture medium thereof had been incubated at 25°C, for 113 days, rotated 60 times/min, with an air flow of 3 liters/min. In mid course, a small quantity of culture solution was added. The culture medium for the jar fermenter was composed as follows: 10 g of malt extract, 10 g of D-glucose, 3.0 g of polypeptone, 3.0 g of yeast extract, 0.1 liter of phosphate buffer solution (M 0.1, p H 5.0), and 0.9 liter of tap water, per liter of medium. After completion of the culture, cultured hyphae were extracted by hot water, and the anti-HIV activity thereof was measured. The hyphae culture solution was filtrated through gauze, to thus obtain hyphae, roughly seven times the volume as H₂O was added, extracted at 90°C for one hour by heating in hot water. The extracts were filtrated through gauze to remove hyphae, filtrated again through a paper filter, then freeze-dried or refrigerated, ready to be used. Testing anti-HIV activity in the same method as described above, the effective dose thereof was determined to be 62.5 µl/ml, showing a high level of activity. In comparison with the activity of this primary processed matter, the present invention showed an activity of 15.6 µl/ml. The primary processed matter obtained by culture after addition of wood substances (lignosulfonic acid) showed an activity of 31.3 µl/ml, and the activity of the present invention extracted thereof was more than 7.3 µl/ml. The activity increased significantly in comparison with the prior technique.

It follows from what has been said that it is possible to obtain cultured products with strong anti-HIV activity or other high physiological activity over a long period, even when a carbon source or other nutrients in a medium become scarce in a liquid

culture (a carbon source mainly using glucose, saccharose, malt sugar, etc.) or in a solid culture (solid culture using sawdust or bagasse as the main ingredients), by adding the nutrients and activators. This has a significant meaning other than incubating for longer duration. According to the present invention, as stated previously in the basic investigation, the way was cleared to obtain the active ingredients in a short period of time, whereby industrial and economic culture methods for Kabanoanatake were shown. However, furthermore, the present invention revealed that long-term cultures are required for some strains to exert activity, depending on the breeds and the properties of Kabanoanatake hyphae, and the above improved culture methods have been established to be applied in such cases. Otherwise, in the case that harvest is not completed within regular hours (on time) according to the convenience of cultivation work, the above improved culture methods have also established techniques to prevent the deterioration of cultured products and the decrease of activity, and to enhance the activity of the cultured products. To sum up, the present invention established industrial methods for short- and long-term cultures of Kabanoanatake. In the culture of Kabanoanatake, however, there were difficulties in artificial culture in that, if the culture medium encountered changes in the composition of culture medium, shaking conditions or temperature, the hyphae died and no active ingredient was produced. In the culture of Kabanoanatake, an epoch-making technique to obtain the present invention was established from the process in which steady harvest of active ingredients can be achieved by addition of wood substances disclosed in this invention to provide resistance to severe changes in conditions and to eliminate the death of hyphae. To clarify the importance, two culture flasks are assumed. If one flask generates active ingredients and if the other flask produces no active ingredient, the activity of active ingredients decreases to a half after mixing the contents of 2 flasks. The risk of decreased quality has been present in the conventional techniques, but this application offered an epoch-making technical process overcoming the weakness. Consequently, a new technique possible to produce the present invention steadily was established.

The inventor took it into consideration that there have been increasingly tragic facts in the world concerning HIV infection after a person survives a sexual assault. If there were a method available to protect from infection by the HIV virus at least in the case of an emergency, it would save women in a vulnerable position from the cruelty of HIV. Namely, in the case that a female that is compelled by force against her will, the inventor invented as a preventive agent or a safety sheath for body insertion in order to prevent HIV infection. Describing in detail, first, 0.1 g of extracted components of

Kabanoanatake was mixed with 3 g of cocoa butter (Fuji Oil Co., Ltd.) as a vehicle. Cocoa butter was put in hot water to heat it up to less than 40°C and melted, whereto extracted components of Kabanoanatake are mixed and agitated. Lowering the temperature to 23°C, cocoa butter is made to crystallize. After that, the crystal is made to melt at 32°C, put in a mold, cooled to solidify, coated with a protective layer, in order to be a product. Anti-HIV activity was determined by the following procedure; this product (containing 0.1 g of effective dose) is inserted in the vagina after a certain length of time, the melted matter of item is wiped off by gauze of 6 × 4 cm, 10 cc of distilled water is added, wrung for collecting the components, thereto added HIV using MT-4 cells, and in the vessel inhibition of growth of HIV was tested. The components being diluted artificially, the results were as follows.

<u>Lapse of time in insertion</u>	<u>Effective dose for HIV inhibition (primary processed matter) (the present invention)</u>	
20 hrs.	1000 µl/ml	400 µl/ml
35 hrs.	1000 µl/ml	400 µl/ml
70 hrs.	1000 µl/ml	400 µl/ml

As evidenced by the results, even after being inserted in the vagina for 70 hours, the active ingredients exerted inhibitory effects against the growth of HIV. Thus, this suppository preventive agent shows great possibility of preventing HIV infection, by carrying this preventive item at an appropriate time when women must go to dangerous areas, or live there, if by any chance, such unexpected incidence should arise. It is of course the case that the present invention is more effective than the primary processed matter. Since the excipient is made of cocoa butter, this preventive agent can be put into the mouth and is edible. This enables the prevention of oral infection with HIV. It is also available in the form of a soluble capsule.

In similar forms such as solid material, soluble capsule, and other infusion of solutions, items containing the present invention can be administered from the vagina or the anus, and items containing the present invention in capsules or chocolates can be taken orally, which may be used for treating HIV-related syndromes including cancer and bacterial disease.

The present invention, originally, can be taken orally, as medicine, either in the form of a powder or in the form of a liquid (ex. solution) and can cure microbe-related syndromes caused by harmful pathogenic bacteria, a species of retroviruses, and adult T-cell leukemia (ATL) and HIV may be inhibited or prevented. There is a case of a 50-year-old Japanese male patient with ATL, who had taken Kabanoanatake extracts

(the present invention) orally for two years and after that recovered good health. Through adding and mixing these extracts in food or beverages, it can be used with daily nutrients as health foods on a daily basis. The concentration added as the standard is not limited to but is 0.1 to 10%. For instance, the present invention can be edible in the form of a powder, solution, or some other form of components of a substance, by adding and mixing them in seasonings such as soy sauce, bean paste, dressing, soup, noodle soup and salt, etc. During production stages, confectionaries such as bread, cake, ice creams, chocolates, frozen desserts, rice cakes, wheat gluten, jelly, gummy, etc or foods such as sandwiches, noodles made of wheat flour, Japanese vermicelli, spaghetti, Chinese noodles, jam, butter and margarine could also be mixed with Kabanoanatake extracts (powder or liquid) to be useful in producing healthy foods. Likewise, during production or when drinking, the present invention can be used in healthy drinks by mixing them with the following foods: meat-processed foods such as sausages, Hamburg steaks, croquettes, tempura, steamed fish paste, seasoned meat, etc; alcohols or alcoholic beverages such as beer, wine, spirits, etc; garlic extract; juice of tomatoes, carrots, mixed vegetable, apple, grapes, etc; carbonated beverage such as cola; fermented tea such as black tea, oolong tea, yanrong tea, etc; yogurt beverages, coffee, green tea, cow milk and cocoa beverages. Furthermore, it is possible to make use of the present invention as folk medicine or health food, through using it as a mixture or concomitantly with Chinese herbal medicines or medicinal plants such as *Cinnamomum sieboldi*, *Lithospermum erythrorhizon* (roots), *Lithospermum officinale* (roots), *Prunus persica*, *Epimedium grandiflorum* var. *thunbergianum*, *Carica papaya* (enzyme), *Ananas nanus* (enzyme), *Panax ginseng*, *Acanthopanax spinosus*, *Poria cocos*, *Ganoderma lucidum*, *Hipsizigus marmoreus*, *Ginkgo biloba* (extract), *Paeonia lactiflora*, *Cnidium officinale*, *Angelicae radix*, etc. In the same way, as a medical treatment, it may be combined with anti-HIV agents such as AZT and DDI.

Artificially cultured Kabanoanatake, especially powder of sawdust cultures extracted by hot water (the primary processed matter and the present invention), exhibit a slightly bitter taste and a spicy savor. By adding this to beer and some other beverages, foods with therapeutic effects can be provided.

Compound 1

NMR spectrum ^1H NMR (CDCl_3) : δ 0.73 (3H, s), 0.81 (3H, s), 0.88 (3H, s), 0.94 (3H, d, $J = 6.5$ Hz), 0.84 (3H, s), 1.00 (3H, s), 1.65 (3H, s), 1.75 (3H, s), 3.24 (1H, dd, $J = 11.8, 4.5$ Hz), 3.67 (1H, ddd, $J = 9.0, 5.2, 4.4$ Hz), 5.18 (1H, m). mass spectrum m/z : 442 (29) $[\text{M}]^+$, 427 (34), 411 (44), 409 (16), 372 (28), 357 (59), 339 (16), 299 (18), 187 (23), 69 (100).

Compound 2

NMR spectrum ^1H NMR (CDCl_3) : δ 0.75 (3H, s), 0.81 (3H, s), 0.89 (3H, s), 0.97 (3H, s), 1.00 (3H, s), 1.59 (3H, s), 1.62 (3H, s), 3.22 (1H, dd, $J = 11.8, 4.8$ Hz). mass spectrum m/z : 456 (60) $[\text{M}]^+$, 441 (66), 423 (100), 395 (11), 301 (10), 281 (17), 187 (19).

Compound 3

NMR spectrum ^1H NMR (CDCl_3) : δ 0.69 (3H, s), 0.81 (3H, s), 0.88 (3H, s), 0.91 (3H, d, $J = 6.5$ Hz), 0.98 (3H, s), 1.00 (3H, s), 3.22 (1H, dd, $J = 11.8, 4.8$ Hz), 5.10 (1H, m). mass spectrum m/z : 442 (77) $[\text{M}]^+$, 427 (62), 409 (100), 391 (26), 357 (15), 327 (10), 299 (10), 273 (11), 259 (11), 255 (10).

Compound 4

NMR spectrum ^1H NMR (CDCl_3) : δ 0.69 (3H, s), 0.81 (3H, s), 0.87 (3H, s), 0.91 (3H, d, $J = 6.5$ Hz), 0.98 (3H, s), 1.00 (3H, s), 3.22 (1H, dd, $J = 11.8, 4.8$ Hz), 4.01 (1H, m), 4.82 (1H, s), 4.93 (1H, s), mass spectrum m/z : 442 (77) $[\text{M}]^+$, 427 (62), 409 (100), 391 (26), 357 (15), 327 (10), 299 (10), 273 (11), 259 (11), 255 (10).

Compound 5

NMR spectrum ^1H NMR (CDCl_3) : δ 0.69 (3H, s), 0.81 (3H, s), 0.90 (3H, s), 0.96 (3H, s), 1.00 (3H, s), 1.57 (3H, s), 1.68 (3H, s), 3.24 (1H, dd, $J = 11.5, 4.4$ Hz), 5.04 (1H, m), 9.46 (1H, d, $J = 5.5$ Hz). mass spectrum m/z : 440 (54) $[\text{M}]^+$, 425 (100), 407 (97), 389 (16), 358 (68), 299 (59), 288 (51), 281 (59), 273 (30), 247 (24).

Compound 6

NMR spectrum ^1H NMR (CDCl_3) : δ 0.73 (3H, s), 0.80 (3H, s), 0.90 (3H, s), 0.97 (3H, s), 0.99 (3H, s), 1.20 (3H, s), 1.22 (3H, s), 3.23 (1H, dd, $J = 11.5, 4.4$ Hz), 3.72 (1H, m). mass spectrum m/z : 458 (45) $[\text{M}]^+$, 443 (32), 425 (100), 407 (90), 389 (13), 299 (45), 281 (28),

Compound 7

NMR spectrum $^1\text{H-NMR}$ (CDCl_3) : δ 0.71 (3H, s, H-18), 0.81 (3H, s, H-29), 0.86 (3H, s, H-30), 0.88 (3H, d, $J = 6.5$ Hz, H-21), 0.98 (3H, s, H-19), 1.00 (3H, s, H-28), 1.05 (1H, dd, $J = 12.8, 3.0$ Hz, H-5), 1.19 (1H, m, H-15 α), 1.22 (3H, s, H-27), 1.23 (3H, s, H-26), 1.23 (1H, td, $J = 13.5, 4.2$ Hz, H-1 α), 1.40 (1H, ddd, $J = 12.0, 10.8, 7.2$ Hz, H-17), 1.48 (1H, m, H-16 β), 1.50 (1H, m, H-6 β), 1.58 (1H, tdd, $J = 13.5, 11.7, 4.2$ Hz, H-2 β), 1.62 (1H, m, H-15 β), 1.65 (1H, m, H-23 β), 1.66 (1H, m, H-2 α), 1.67 (1H, m, H-6 α), 1.70 (1H, m, H-12), 1.73 (1H, td, $J = 13.5, 4.2$ Hz, H-1 β), 1.83 (1H, m, H-16 α), 1.83 (1H, d quint., $J = 12.0, 6.7$ Hz, H-20), 2.01 (1H, m, H-11), 2.01 (1H, m, H-23 α), 2.04 (1H, m, H-7), 3.23 (1H, dd, $J = 11.7, 4.6$ Hz, H-3), 3.92 (1H, dd, $J = 6.4, 4.1$ Hz, H-24), 4.26 (1H ddd, $J = 10.3, 6.6, 3.7$ Hz, H-22). $^{13}\text{C-NMR}$ (CDCl_3) : δ 12.3 (C-21), 15.4 (C-29), 15.7 (C-18), 18.2 (C-6), 19.1 (C-19), 21.0 (C-11), 21.2 (C-26), 24.3 (C-30), 26.5 (C-7), 27.3 (C-16), 27.5 (C-27), 27.8 (C-2), 28.0 (C-28), 30.9 (C-12), 31.0 (C-15), 33.3 (C-23), 35.6 (C-1), 37.0 (C-10), 38.5 (C-20), 38.9 (C-4), 45.0 (C-13), 47.8 (C-17), 49.3 (C-14), 50.4 (C-5), 78.1 (C-22), 78.5 (C-24), 79.0 (C-3), 81.7 (C-25), 134.2 (C-8), 134.5 (C-9). mass spectrum m/z : 458 (52) $[\text{M}]^+$, 443 (75), 425 (66), 407 (11), 339 (17), 314 (10), 311 (10), 301 (11), 283 (12), 115 (100), 71 (68).

Compound 8

NMR spectrum $^1\text{H-NMR}$ (CDCl_3) : δ 0.72 (3H, s, H-18), 0.81 (3H, s, H-29), 0.87 (3H, s, H-30), 0.93 (3H, d, $J = 6.7$ Hz, H-21), 0.98 (3H, s, H-19), 1.00 (3H, s, H-28), 1.05 (1H, dd, $J = 12.5, 2.5$ Hz, H-5), 1.18 (1H, m, H-15 α), 1.23 (1H, td, $J = 12.6, 3.8$ Hz, H-1 α), 1.41 (1H, m, H-16 β), 1.44 (1H, ddd, $J = 12.7, 9.6, 7.3$ Hz, H-17), 1.50 (1H, m, H-6 β), 1.58 (1H, m, H-2 β), 1.58 (1H, m, H-23 β), 1.63 (1H, m, H-15 β), 1.67 (1H, dqd, $J = 12.7, 9.6, 3.2$ Hz, H-20), 1.67 (1H, m, H-23 α), 1.68 (1H, m, H-2 α), 1.68 (1H, m, H-6 α), 1.68 (1H, m, H-12 β), 1.73 (1H, m, H-12 α), 1.73 (3H, s, H-27), 1.74 (1H, dt, $J = 12.6, 3.8$ Hz, H-1 β), 1.78 (1H, m, H-16 α), 2.01 (1H, m, H-11), 2.04 (1H, m, H-7), 3.23 (1H, dd, $J = 11.8, 4.5$ Hz, H-3), 3.98 (1H ddd, $J = 10.2, 3.2, 2.8$ Hz, H-22), 4.36 (1H, t, $J = 4.8$ Hz, H-24), 4.94 (1H, q, $J = 1.8$ Hz, H-26A), 5.11 (1H, q, $J = 1.2$ Hz, H-26B). $^{13}\text{C-NMR}$ (CDCl_3) : δ 12.6 (C-21), 15.4 (C-29), 15.7 (C-18), 18.2 (C-6), 19.1 (C-19), 19.3 (C-27), 21.0 (C-11), 24.3 (C-30), 26.5 (C-7), 27.2 (C-16), 27.8 (C-2), 28.0 (C-28), 31.0 (C-12), 31.0 (C-15), 33.1 (C-23), 35.6 (C-1), 37.0 (C-10), 38.9 (C-4), 42.8 (C-20), 44.8 (C-13), 47.2 (C-17), 49.4 (C-14), 50.4 (C-5), 70.3 (C-22), 73.5 (C-24), 79.0 (C-3), 110.2 (C-26), 134.1 (C-8), 134.6 (C-9), 147.3 (C-25). mass spectrum m/z : 458 (9) $[\text{M}]^+$, 357 (75), 339 (18), 311 (13), 159 (10).

Compound 9

NMR spectrum ^1H -NMR (CDCl_3) : δ 0.72 (3H, s, H-18), 0.81 (3H, s, H-29), 0.87 (3H, s, H-30), 0.94 (3H, d, $J = 6.7$ Hz, H-21), 0.98 (3H, s, H-19), 1.00 (3H, s, H-28), 1.05 (1H, dd, $J = 13.0, 2.3$ Hz, H-5), 1.21 (1H, ddd, $J = 14.2, 9.3, 2.5$ Hz, H-15 α), 1.23 (1H, td, $J = 12.6, 3.8$ Hz, H-1 α), 1.45 (1H, m, H-16 β), 1.46 (1H, ddd, $J = 12.7, 9.6, 7.3$ Hz, H-17), 1.51 (1H, m, H-6 β), 1.52 (1H, m, H-23 β), 1.54 (1H, m, H-23 α), 1.58 (1H, qd, $J = 13.5, 3.2$ Hz, H-2 β), 1.63 (1H, m, H-15 β), 1.67 (1H, m, H-2 α), 1.68 (1H, m, H-6 α), 1.70 (1H, dqd, $J = 12.7, 6.7, 3.0$ Hz, H-20), 1.69 (1H, m, H-12 β), 1.71 (1H, m, H-12 α), 1.74 (1H, dt, $J = 12.6, 3.8$ Hz, H-1 β), 1.76 (3H, s, H-27), 1.78 (1H, m, H-16 α), 2.02 (1H, m, H-11), 2.04 (1H, m, H-7), 3.24 (1H, dd, $J = 11.7, 4.5$ Hz, H-3), 3.96 (1H dt, $J = 9.2, 3.0$ Hz, H-22), 4.26 (1H, dd, $J = 9.2, 3.5$ Hz, H-24), 4.84 (1H, dq, $J = 3.8, 1.8$ Hz, H-26A), 5.01 (1H, quint., $J = 1.2$ Hz, H-26B). ^{13}C -NMR (CDCl_3) : δ 12.7 (C-21), 15.4 (C-29), 15.8 (C-18), 17.9 (C-27), 18.2 (C-6), 19.1 (C-19), 21.0 (C-11), 24.4 (C-30), 26.5 (C-7), 27.2 (C-16), 27.8 (C-2), 28.0 (C-28), 30.9 (C-12), 30.9 (C-15), 34.6 (C-23), 35.6 (C-1), 37.0 (C-10), 38.9 (C-4), 42.5 (C-20), 44.8 (C-13), 47.3 (C-17), 49.4 (C-14), 50.4 (C-5), 74.6 (C-22), 76.5 (C-24), 79.0 (C-3), 110.8 (C-26), 134.1 (C-8), 134.6 (C-9), 147.6 (C-25). mass spectrum m/z : 458 (9) $[\text{M}]^+$, 357 (75), 339 (18), 311 (13), 159 (10).

Compound 10

NMR spectrum ^1H NMR (CDCl_3) : δ 0.70 (3H, s), 0.81 (3H, s), 0.88 (3H, s), 0.92 (3H, d, $J = 6.5$ Hz), 0.98 (3H, s), 1.00 (3H, s), 3.24 (1H, dd, $J = 11.2, 4.6$ Hz), 3.62 (2H, m). mass spectrum m/z : 402 (37) $[\text{M}]^+$, 387 (100), 369 (90), 273 (16), 187 (27).

Compound 11

NMR spectrum ^1H NMR (CDCl_3) : δ 0.72 (3H, s), 0.81 (3H, s), 0.90 (3H, s), 0.99 (3H, s), 1.00 (3H, s), 1.61 (3H, s), 1.69 (3H, s), 3.24 (1H, dd, $J = 11.7, 4.3$ Hz), 3.68 (1H, dd, $J = 11.2, 4.6$ Hz), 3.73 (1H, dd, $J = 11.2, 3.0$ Hz), 5.12 (1H, m). mass spectrum m/z : 442 (57) $[\text{M}]^+$, 427 (49), 409 (28), 189(12), 187 (11), 109(100).